NEURAL RESPONSES TO INJURY: PREVENTION, PROTECTION, AND REPAIR
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Submitted by

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Neurochemical Protection of the Brain, Neural Plasticity and Repair

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Neurochemical Protection of the Brain, Neural Plasticity and Repair

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TRAUMATIC BRAIN INJURY

INTRODUCTION

Traumatic brain injury is characterized by multiple phases of damage; including primary tissue damage and bleeding at the site of impact; secondary damage, including brain edema, ischemia, the diffusion of toxic substances beyond the initial site of injury and delayed neuronal death; and long-term epileptogenic changes in synaptic plasticity. A common motif at the cellular level of these various forms of neurotrauma is the over-release of neurotransmitters, the stimulation of post-synaptic receptors, and the subsequent accumulation of abnormally high concentrations of second messengers. The major neurotransmitter involved in neuronal damage is thought to be the excitatory amino acid L-glutamate. Glutamate triggers calcium entry into post-synaptic neurons via the N-methyl-D-aspartate (NMDA) subclass of glutamate receptors (Rothman and Olney, 1986, 1987; Choi 1988), and so the activation of many calcium-dependent signaling pathways.

The major focus of research in our laboratory has been the activation of calcium-dependent phospholipases A_2, the release from membrane phospholipids of bioactive lipids, including free arachidonic acid (AA) and platelet-activating factor (PAF), and the signaling pathways then activated. We and other groups have shown the neuroprotective properties of pharmacological agents targeting bioactive lipid signaling cascades. Detailed characterization of these processes, and the downstream events that link the over-accumulation of bioactive lipids to long-term changes in brain physiology, is important in identifying the best therapeutic targets for the treatment of traumatic brain injury.

Free AA can be metabolized to several well-described classes of bioactive lipids, including prostaglandins (PG), leukotrienes (LT), and thromboxanes (TX) or can by itself exert

effects on synaptic activity. The rate-limiting step in the biosynthesis of PG and TX (collectively known as prostanoids) is the conversion of arachidonic acid to PGH_2. This reaction is catalyzed by cyclooxygenase (COX, prostaglandin G/H synthase, PGS). One of the most important recent findings in the cell biology of bio active lipids is the discovery of a second isoform of the enzyme, COX 2. Expression of the two isoforms is regulated in distinct ways. In most tissues, COX 1 is constitutively expressed, whereas COX 2 expression is stimulated by growth factors and cytokines, and displays a pattern of induction typical of the product of an immediate-early gene (Herschman, 1996).

The biological needs for two different isoforms of COX are not immediately apparent, given that both catalyze the identical reaction, and display similar kinetics. COX 1 is generally thought to be responsible for the biosynthesis of constitutively required prostanoids, whereas COX 2 induction enhances the biosynthesis of prostanoids under stimulated conditions. However, the roles of COX 2 may be more complex. Given that PLA_2 is rapidly activated (1-2 minutes), but that COX 2 protein levels are usually not elevated until 1-2 hours after a given stimulus, then the AA released within minutes of a stimulus is probably used by COX 1, whereas COX 2 uses substrate pools accumulated hours after the initial stimulus. Additionally, whereas COX 1 protein expression is confined to the endoplasmic reticulum, COX 2 has also been found in nuclear membranes (Morita et al, 1995; Coffey et al, 1997). COX 2 may therefore use arachidonic acid pools distinct from those metabolized by COX 1, and may also be responsible for the synthesis of prostanoids involved in nuclear signalling events.

The brain is one of the few sites in the body where COX 2 expression is detectable under resting conditions. This seemingly constitutive expression, which is localized in neurons, is probably accounted for by activity-dependent induction in excitatory neurons (Yamagata et al,

1993). COX 2 immunoreactivity in brain has been noted in the post-synaptic structures of discrete subpopulations of excitatory neurons from cerebral cortex, hippocampus and amygdla (Kaufmann et al, 1996). Normal synaptic activity, specifically that involving the NMDA class of glutamate receptors, is sufficient to stimulate COX 2 expression (Yamagata et al, 1993). Therefore, the resting levels of COX 2 in brain can be accounted for by the proportion of excitatory neurons undergoing stimulation at any given moment.

Besides being regulated by synaptic activity, COX 2 expression in the brain is also regulated by the pro-inflammatory cytokines released by astrocytes, microglia, and infiltrating inflammatory cells. COX 2 may therefore have a pivotal role at various stages of traumatic brain injury, including primary tissue damage, secondary inflammation and edema, recruitment of surrounding tissue into either degenerative or reparative processes, and the increased neuronal excitability associated with epileptogenesis.

We and others have previously shown that COX 2 mRNA and protein are strongly upregulated during several models of neurotrauma, including vasogenic brain edema (Bazan et al, 1996, and previous annual reports on this project) electroconvulsive shock (ECS), kainic acid (KA)-induced seizures (Marcheselli and Bazan, 1996), focal ischemia-reperfusion (Planas et al, 1995; Collaco-Moraes et al, 1996), and spreading depression (Miettinen, 1997). Several lines of evidence points to a role for COX 2 in neuronal damage, (I) In ischemia reperfusion (Collaco-Moraes et al, 1996) and seizure models (Marcheselli and Bazan, 1996), COX 2 expression is upregulated in those brain regions most vulnerable to damage; (ii) COX 2, but not transcription factor immediate-early genes, are more highly upregulated in damaging versus non-damaging seizures; (iii) the specific COX 2 enzyme inhibitor NS 398 reduces infarct volume in a model of focal ischemia-reperfusion (Nogawa et al, 1997).

We have shown that PAF can activate transcriptional activity from the COX 2 promoter (Bazan et al, 1994), and that the PAF receptor antagonist BN 50730 inhibits COX 2 induction in the ECS, KA and vasogenic edema models of brain trauma (Marcheselli and Bazan, 1996; Bazan et al, 1996). This suggests that PAF synthesized because of short-term activation of $cPLA_2$, such as induced via NMDA receptor activation, can influence downstream events in the injury/repair cascade by mechanisms that involve the activation of COX 2.

Transcription of the COX 2 gene is regulated by a complex set of transcription factors and other DNA binding proteins that associate with the most-proximal region of the promoter. As detailed below, although the nucleotide sequence of this part of the COX 2 gene promoter has diverged considerably between mouse, rat and human, several transcription factor binding sites, most notably the NFκB and AP2 DNA binding consensus sequences, remain highly conserved among these three species. The most important of these is NFκB, which been identified as crucial transcription factor for neuronal and glial cell function (O'Neill and Kaltschmidt, 1997). NFκB activity is upregulated by traumatic brain injury (Yang et al, 1995), cerebral ischemia (Salminen et al, 1995), seizures (Rong and Baudry, 1996), and several neurodegenerative diseases (O'Neill and Kaltschmidt, 1997). Unlike many other transcription factors such as fos, jun and zinc-finger proteins, the expression of which is stimulated via transcriptional activation, NFκB exists as a preformed, heterodimeric complex. In its inactive form it resides in the cytoplasm complexed to an inhibitor protein IκB. Various stimuli activate a yet undefined kinase that phosphorylates IκB. NFκB then dissociates from IκB and is translocated to the nucleus, where it activates the transcription of target genes containing NFκB response elements in their promoters. IκB is ubiquinated and degraded by the proteasome. Several different NFκB and IκB proteins have been identified.

The COX 2 gene is rapidly and transiently induced by traumatic injury to the brain, such as after cryogenic injury, electroconvulsive shock, or after the administration of arachidonic acid cascade stimulating agents (Ca^{2+}, Mg^{2+}, zymosan), lipopolysaccharide, or after treatment with kainic acid (KA) or platelet activating factor (PAF; 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phospho-choline; 2-11). Importantly, COX 2 gene induction can be inhibited by hetrazepines such as BN50730, a PAF antagonist that is selective for intracellular PAF binding sites (Marcheselli and Bazan, 1996), or by the anti-inflammatory and immuno-suppressive glucocorticoid dexamethasone, via an IKBa-NFκB regulatory loop (Auphan et al, 1995; Scheinman et al, 1995).

This report summarizes our efforts to characterize protein-DNA interactions, especially AP2 and NFκB binding, within the immediate upstream regulatory regions of the COX 2 gene in the uninduced (resting) state and in PAF-induced NG108-15 (mouse neuroblastoma x rat glioma) cultured cells, in IM9 (human lymphoblast) tissue culture cells, in the rat hippocampus isolated after CI, ECS or KA treatment, and in normal human hippocampus. The techniques of promoter deletion analysis, electrophoretic mobility shift assays (EMSA), magnetic capture of DNA-binding proteins and ultrasensitive silver staining (0.2 ng/mm^2) was used to characterize COX 2 promoter protein-DNA interactions.

BODY

Previous work

In previous reports (**years 1 & 2**), we have shown that COX 2 mRNA and protein levels are up-regulated during cryogenically-induced vasogenic brain edema in rats. COX 2 protein levels peak at 4-5 hours post-injury, 2-3 hours after the peak in COX 2 mRNA levels. The induction is sustained up to 24 hours post-injury. A role for platelet-activating factor in COX 2 activation was implied by the ability of the specific intracellular PAF receptor antagonist BN 50730, and the novel PAF antagonist LAU 503, to block this induction at least partially. PAF receptor antagonists and glucocorticoids (inhibitors of COX 2 up-regulation) also reduce blood-brain barrier breakdown in this model, implying a role for PAF-induced COX 2 activation in edema formation. We used electrophoretic mobility shift assays (EMSAs) to show that the DNA binding activities from rat brain nuclear extracts specific for AP2, CREB, GAS/ISRE and NFκB decreased during the cryogenic injury. We presented evidence of a novel DNA binding protein in rat brain nuclear protein extracts which binds to a region (-460 to -258) of the COX 2 promoter. This protein was present only in uninjured brains, and disappeared during cryogenic injury. We speculated that this protein represented a repressor of COX 2 expression, the removal of which during injury was an important step in the increase of COX 2 transcriptional activity.

Objectives, year 4

We wanted to expand on our studies of transcription factor regulation during the brain edema model in the rat by identifying the factors, specifically induced by PAF, that regulate COX 2 promoter activity. This entailed using cell lines in which PAF induces COX 2 transcription. During this project, sequence data for the COX 2 promoter from other species

(mouse and human) became available, and there were apparently interspecies variations in promoter structure. Therefore, if COX 2 gene activation were to be a potentially useful therapeutic target, finding it out whether the factors important in COX 2 promoter activation in our rat model were similar to those activating the human promoter was necessary.
For these studies, using for our EMSAs nuclear protein extracts from cell lines in which PAF induces COX 2 expression was necessary. We used a rat glioma/mouse neuroblastoma hybrid cell line (NG108-15), and a human B lymphoblast line (IM9) as our PAF-responsive cell lines.

We also wanted to characterize the putatative rat COX 2 repressor protein further, and to learn if a homologous protein was involved in human COX 2 promoter activity. For this we used DNA-magnetic bead affinity technology, designed to isolate and purify proteins that bind to a specific DNA sequence.

Materials and Methods

Tissue Culture Cell and Preparation of Nuclear Protein Extracts (NPXTs)

NG108-15 (mouse neuroblastoma x rat glioma hybrids) or IM9 (human b-lymphoblasts) cells were incubated with PAF (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) to induce transcription of the COX 2 gene (Bazan et al., 1994); six week old Wistar rats were subjected to either a single CI or ECS, or were treated with KA, to induce COX 2 transcription. NPXTs were prepared from cells in tissue culture or isolated whole hippocampus (Lukiw et al, 1994).

Preparation and Radiolabelling of COX 2 Promoter DNA

DNA was synthesized by PCR using human-specific COX 2 primers (Table 1) and human genomic DNA as template. For EMSA, COX 2 promoter DNA fragments were 5' end-labelled using γ-^{32}P ATP (Amersham); purified transcription factors AP1, AP2, C/EBP, CRE-1, CREB, EGR1(zif268), GAS/ISRE, GATA, NFκB, OCT, SIE, SP1 and SRE, and competitor oligonucleotides for TFIID, SP1, AP1, AP2, AP3, CTF/NF1, NFκB and OCT1 were purchased from Promega (Madison, WI).

Electrophoretic Mobility Shift Assay (EMSA)

EMSA was performed as described in (Lukiw et al, 1994); typically 5-20 ug NPXTs from control or COX 2 induced samples were added to labeled DNA (~50,000 cpm). EMSA samples were analyzed on acrylamide/glycerol composite gels; gels were dried and autoradiographed using Kodak BioMax MR film or phosphor-screen stored images were scanned using a Bio-Rad

GS250 Molecular Imager and analyzed with their Molecular Analyst software package.

Isolation of DNA-Binding Proteins by Magnetic Bead Capture and Silver Stain Analysis on TGSDS-PAGE Gels

DNA fragments of the COX 2 promoter, singly end-labelled with biotin by using singly biotinylated PCR primers (Figure 1), were coupled to strepavidin-coated M-280 Dynabeads (Dynal^{R}) and were incubated with NPXTs; after washing, the magnetically separated beads (containing COX 2 promoter DNA-binding proteins) were placed into a Tris-Glycine-SDS/PAGE (TGSDS-PAGE) sample buffer (62.5 mM Tris-HCl pH 7.5, 10% glycerol, 2% (w/v) SDS, 2% (w/v) b-mercaptoethanol, 0.025% (w/v) bromophenol blue, overlaid with mineral oil, heated 5 minutes at 95°C in 0.5 ml PCR reaction tubes and were applied directly to a 0.75 mm thick 4-20% TGSDS/PAGE gradient gel (BioRad Mini PROTEAN II Cell) and were stained with silver using the BioRad silver stain plus kit.

Primer Design, Multiplex RT-PCR and Automated DNA Sequence Analysis

All DNA sequence computer searches were performed utilizing ENTREZ Version 3.010 and the NCBI DNA databank Version 15.0, OLIGO Version 5.0 for multiplex both standard and RT-PCR (MPX RT-PCR) primer design and selection and the Kodak IBI Pustell DNA Sequence analysis package for DNA restriction and structure analysis. Multiplex (MPX) RT-PCR was performed as described by Lukiw et al, 1994.

RESULTS

Transcription factor binding sites in the rat, mouse and human COX 2 promoters were mapped using a combination of software DNA sequence analysis and EMSA. For the software analysis, the promoter sequences were scanned for known transcription factor binding consensus sequences. In the EMSA studies, specific regions of the promoters were synthesized using PCR, and tested for their ability to bind individual purified transcription factor proteins. The results of these studies are summarized in Figures 1 and 2, and in Table 1. Several species differences in promoter structure were noted. While the presence of AP2 and NFκB elements, binding sites for transcription factors shown in our previous studies to be downregulated during brain edema, were conserved between species, their number and position were not. In addition, specific binding sites were lost (i.e., CRE in rat) or gained (I. e. a conventional TATA box in human).

We had previously shown that PAF transcriptionally activates the COX 2 promoter, and that BN 50730 inhibits this response in the mouse neuroblastoma x rat glioma hybrid cell line NG108-15 (Bazan et al, 1994). These findings made this cell line a suitable model for studying which transcription factors are involved in PAF induction of COX 2 transcription. However, in the light of the species differences in COX 2 promoter structure, studying was necessary in addition and compare the PAF response in a human cell line. The cell line we used was the human B lymphoblast line IM9, a line shown to be PAF-responsive (Nguer et al, 1992). We used multiplex RT-PCR to show that PAF elevates levels of mRNA for COX 2, but not COX 1, and that the PAF antagonist BN 50730 inhibits the induction of COX 2 (Figure 3).

Intracerebroventricular injection of rats with BN 50730 before the stimulus inhibits the

seizure-induced up-regulation of COX 2 expression in hippocampus (Figure 4). EMSA of NPXTs from the hippocampi of these animals shows that BN 50730 inhibits the downregulation of transcription factor activities triggered by seizures (Figure 5), similar to our observations using the brain edema model (Progress Report, year 2). Treatment of IM9 cells with PAF induces a similar pattern of downregulation of the transcription factors AP-2, CRE and NFκB, which is sensitive to BN 50730 (Figure 6). Thus, the PAF response in the human cell line *in vitro* is qualitatively similar to that of the BN 50730-sensitive response to neurotrauma in the rat.

The ability of PAF to induce the injury-associated nucleoprotein identified in the brain edema model (Progress Report, year 2) was assessed in the cell lines. Nucleoprotein extracts were prepared from NG108-15 and IM9 cells treated for different times with PAF. Proteins binding to the mouse and human COX 2 promoter sequences were then isolated by magnetic bead capture technology and resolved by SDS polyacrylamide gel electrophoresis. In each case the disappearance of a 32-40 kDa protein upon COX 2 gene induction (arrow) that is present only in the resting (uninduced) state was noted (Figure 7).

DISCUSSION

1. As determined by EMSA and transfection studies, the COX 2 gene is modulated by a complex series of DNA binding proteins (Figures 1 and 2), and in particular AP-2, NFκB and several yet unidentified regulatory elements that include a set of gene repressor proteins. Elevations in the protein-DNA binding of AP2 and NFκB immediately precede maximal COX 2 activation in IM9 cells. The preferential blocking of induction of transcription factors AP2 and NFκB, but neither zif-268 nor the GAS/ISRE element using the PAF antagonist BN50730 suggests that AP2 and NFκB are intimately linked with PAF-mediated gene induction.

2. As determined by MPX RT-PCR using PCR primers specific for β-actin, COX1 and COX 2, the COX 2 gene is rapidly activated in human IM9 cells (FIGURE 3). Inhibition of COX 2 gene induction by BN50730 is probably the result of a pathway involving AP2 and NFκB. BN50730 may arrest the mechanism by which cytoplasmic NFκB is activated and translocated to the nucleus to stimulate the transcription of NFκB-sensitive genes such as COX 2.

3. The time course of binding of transcription factors NFκB and AP2 and BN50730-mediated inhibition of this protein-DNA interaction suggests an ordered sequence of events in COX 2 activation as mediated by these two positively acting transcription factors. Temporally, these events immediately follow the leaving of ~32-40 kDa repressor protein(s) from the COX 2 promoter.

4. Utilizing DNA-magnetic bead affinity technology it was possible to show one ~32-40 kDa DNA-binding protein leaves the COX 2 gene promoter region during the process of gene induction, and therefore in the resting or uninduced state may act as a COX 2 gene repressor. We propose that removal of this non histone chromosomal protein (NHCP) protein from the COX 2 gene promoter may allow other positively activating transcription factors such as NFκB to bind in the place of, or near, this regulatory protein to activate transcription from the COX 2 gene locus. The details of this proposed mechanism, and how secondary DNA structures such as stem-loops and palindromes in the immediate COX 2 promoter might be involved in this regulation are currently under investigation.

6. Although the rat and human COX 2 promoters show some differences in structure, the important features of the mechanisms by which PAF regulates their activity appear, at this stage, to be similar. Thus, we predict that our findings in rat models of neurotrauma can be used to help identify potential human therapeutic targets for brain trauma directed at COX 2 activation.

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FIGURES AND TABLES

FIGURE LEGENDS

FIGURE 1A

Transcription factors and other modulatory elements lying between -500 and +134 bp of the mouse, rat and human COX 2 promoters. Note the conservation of the presence of the transcription regulatory elements AP2 and NFκB, but not of their number nor position in the COX 2 promoter from the mouse, rat and human genome. In addition, specific transcription factor binding sites have been lost (i.e. CRE in rat) or gained (i.e. a conventional TATA box in humans). Futher details on the fine chromatin structure and regulatory elements located within the human COX 2 promoter and PCR primer / COX 2 promoter DNA fragment selection are depicted in FIGURE 1B

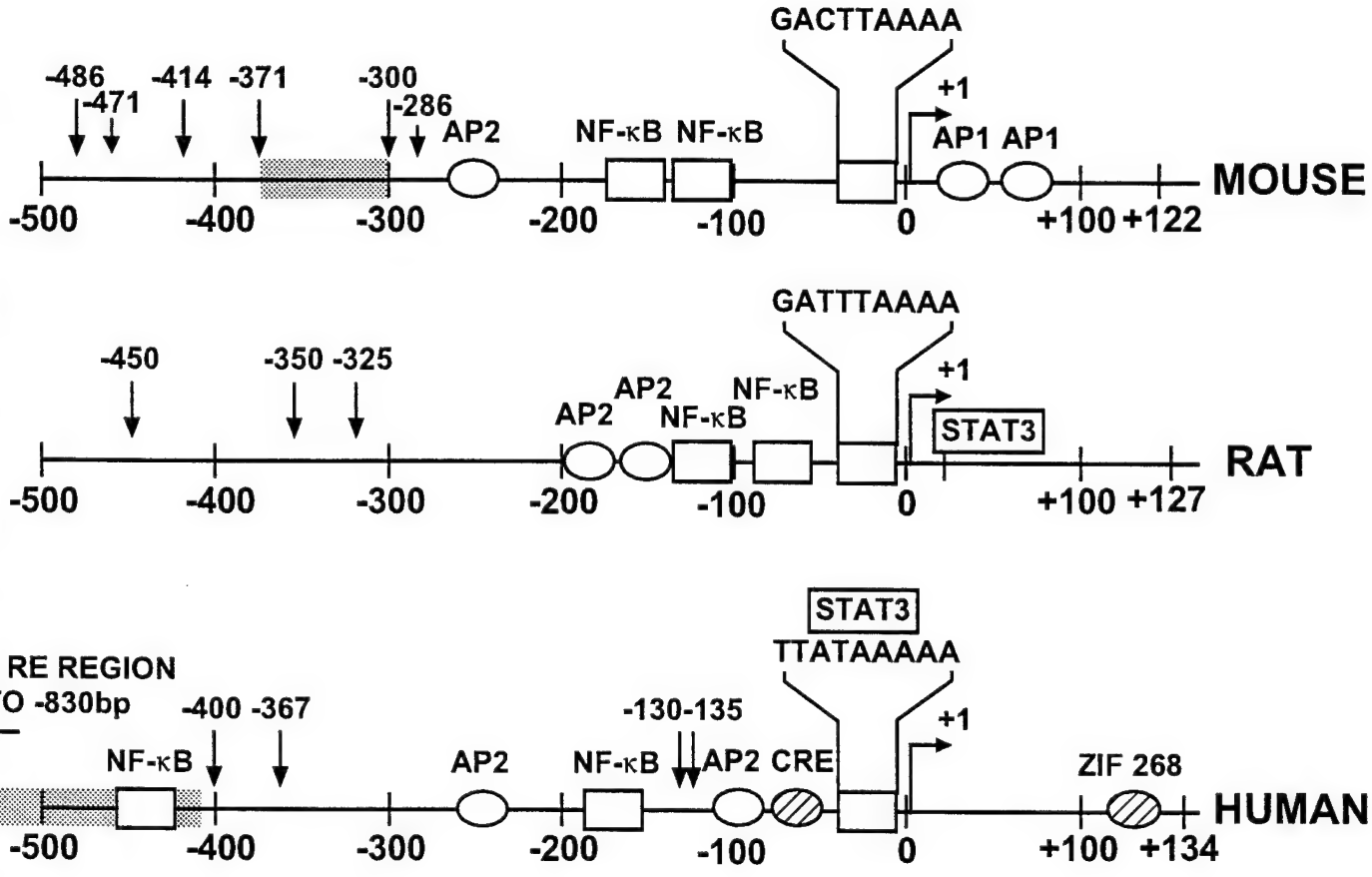
GACTTAAAA
-486
-471
-414
-371
-300
-286
AP2
NF-κB NF-κB
+1
AP1 AP1
MOUSE
-500
-400
-300
-200
-100
0
+100 +122
GATTTAAAA
-450
-350 -325
AP2
AP2
NF-κB
NF-κB
+1
STAT3
RAT
-500
-400
-300
-200
-100
0
+100 +127
STAT3
TTATAAAAA
PAF RE REGION
TO -830bp
-400 -367
-130-135
+1
NF-κB
AP2
NF-κB
AP2 CRE
ZIF 268
HUMAN
-500
-400
-300
-200
-100
0
+100 +134

FIGURE 1B

Transcription factor consensus sequences and other modulatory elements lying between -1000 and +165 bp of the human COX 2 promoter.

TRANSCRIPTION FACTORS AND OTHER REGULATORY ELEMENTS IN THE PAF RESPONSE ELEMENT (PAFRE) IN THE HUMAN COX-2 GENE PROMOTER

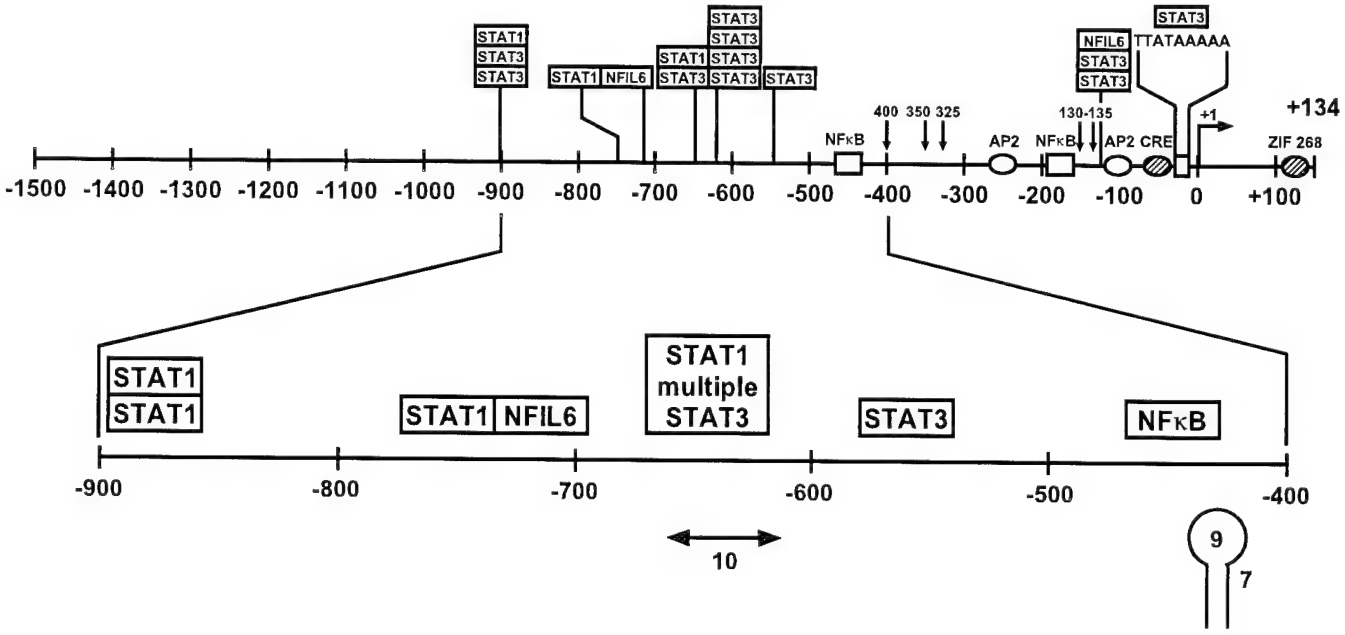

Figure 1B
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FIGURE 2

Major modulatory elements lying between -1000 bp and +165 bp of the human cyclooxygenase (COX 2) promoter

(Major modulatory elements lying between -1000 bp and +165 bp of the human cyclooxygenase (COX 2) promoter)

ELEMENT:	DNA SEQUENCE	POSITION
PALINDROME*:	5'-ATGCCTTAAGGCAT-3'	(-939 to -926 bp)
STAT3:	5'-TT[N_4]AA-3'; 5'-TTCCACAA-3'	(-646 to -532 bp)
PALINDROME:	5'-TTTTTAAAAA-3'	(-629 to -620 bp)
STEM-LOOP*:	5'-GCGGCCCCGGACCTCAGGGCCGC-3'	(-437 to -415 bp)
NFKB*p*** :	5'-AGAGTGGGGACTACCCCCT CT-3'	(-229 to -209 bp)
NFKB*d*** :	5'-GGAGAGGGGATTCCCTGCGCC-3'	(-452 to -433 bp)
AP2*p*:	5'-GCCCCCACCGGG-3'	(-145 to -134 bp)
AP2-like:	5'-GAACTCGGGGAGGA-3'	(-262 to -249 bp)
NFIL6:	5'-TKNNGNAAK-3';5'-TTACGCAAT-3'	(-133 to -122 bp)
STAT3:	5'-TTACGCAA-3'	(-133 to-126 bp)
STAT3:	5'-TTTTTTAA-3'	(-124 to -117 bp)
CRE:	5'-TTTCGTCA-3'	(-60 to -53 bp)
STAT1:	5'-TT[N_5]AA-3'; 5'-TTATAAAAA-3'	(-32 to -25 bp)
TATA BOX:	5'-TTATAAAAA-3'	(-32 to -24 bp)
TFIID:	5'-TATA-3'	(-31 to -28 bp)
TRXN START**:	5'-AGCGACCAATTGTC-3'	(-7 to +7 bp)
CpG ISLAND***:	5'-CCCCCGCGCCGCGCCCTGCCCGCCGC-3'	(+105 to+130 bp)
ATG START:	5'-ATG-3'	(+136 to +138 bp)
zif-268 (Egr1):	5'-GTGCGCGG-3'	(+158 to +165 bp)

*only palindromes and stem-loop motifs of stabilities $\Delta G \leq$ -14.6 kcal/mol are shown.

** relevant "core" sequence or specific nucleotide are double underlined.

***CpG dinucleotides are double underlined.

FIGURE 3

Multiplex RT-PCR using the primer sets for β-actin, COX1 and COX 2 in PAF-induced IM9 cells.

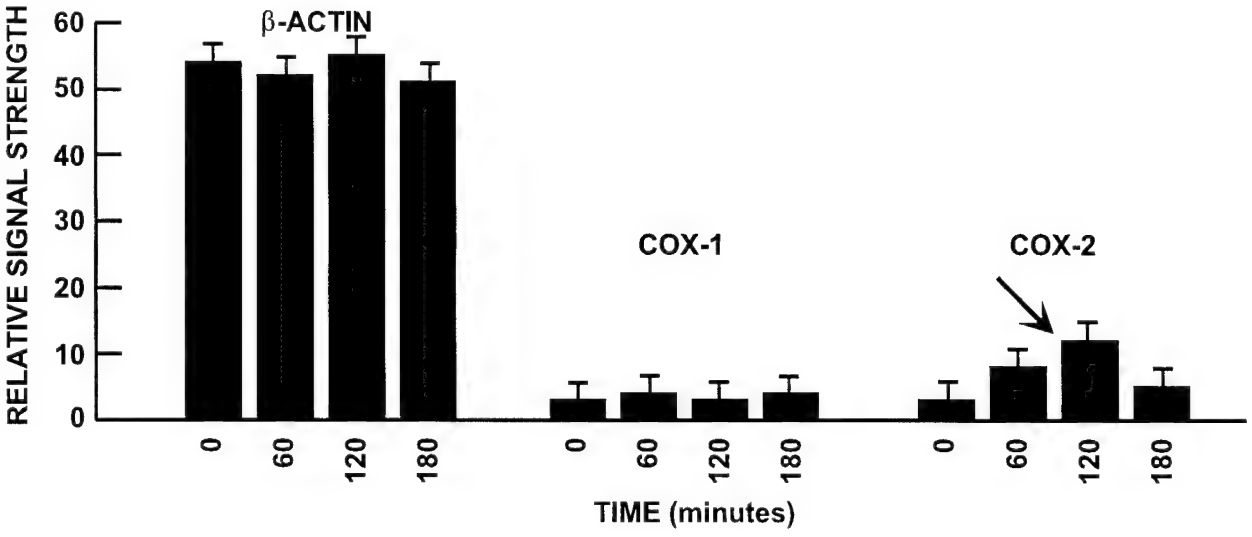
RELATIVE SIGNAL STRENGTH
60
50
40
30
20
10
0
β-ACTIN
COX-1
COX-2
0
60
120
180
0
60
120
180
0
60
120
180
TIME (minutes)

Figure 3
Bazan, Chapter 4, 1997

FIGURE 4

BN50730- and dexamethasone-mediated inhibition of COX 2 gene expression during seizure. The hetrazepine BN50730, an antagonist of intracellular PAF binding sites, and the glucocorticoid dexamethasone, have each been shown to strongly inhibit expression of the COX 2 gene after induced seizures in rats (Marcheselli and Bazan, 1996; Bazan et al, 1996).

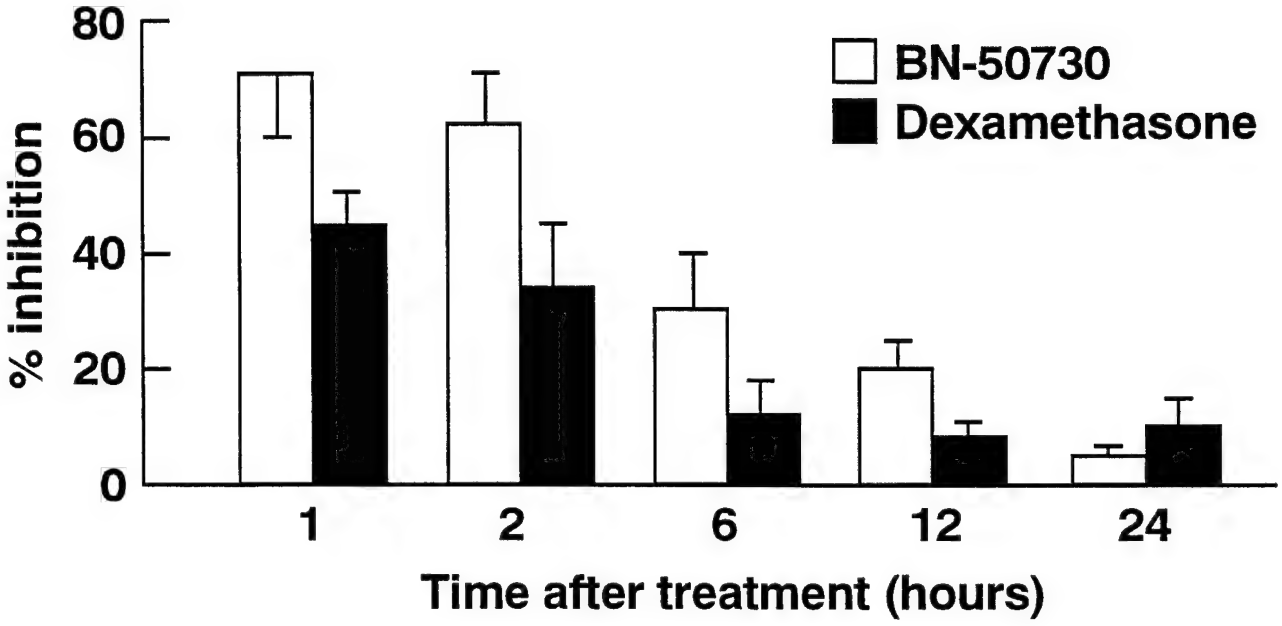
80
60
40
20
0
% inhibition
BN-50730
Dexamethasone
1
2
6
12
24
Time after treatment (hours)

FIGURE 5

Electrophoretic mobility shift assay (EMSA) of rat hippocampal nuclear protein extracts (NPXTs) and the AP2, zif-268, GAS/ISRE, NFKB and NFIL-6 DNA consensus sequences.

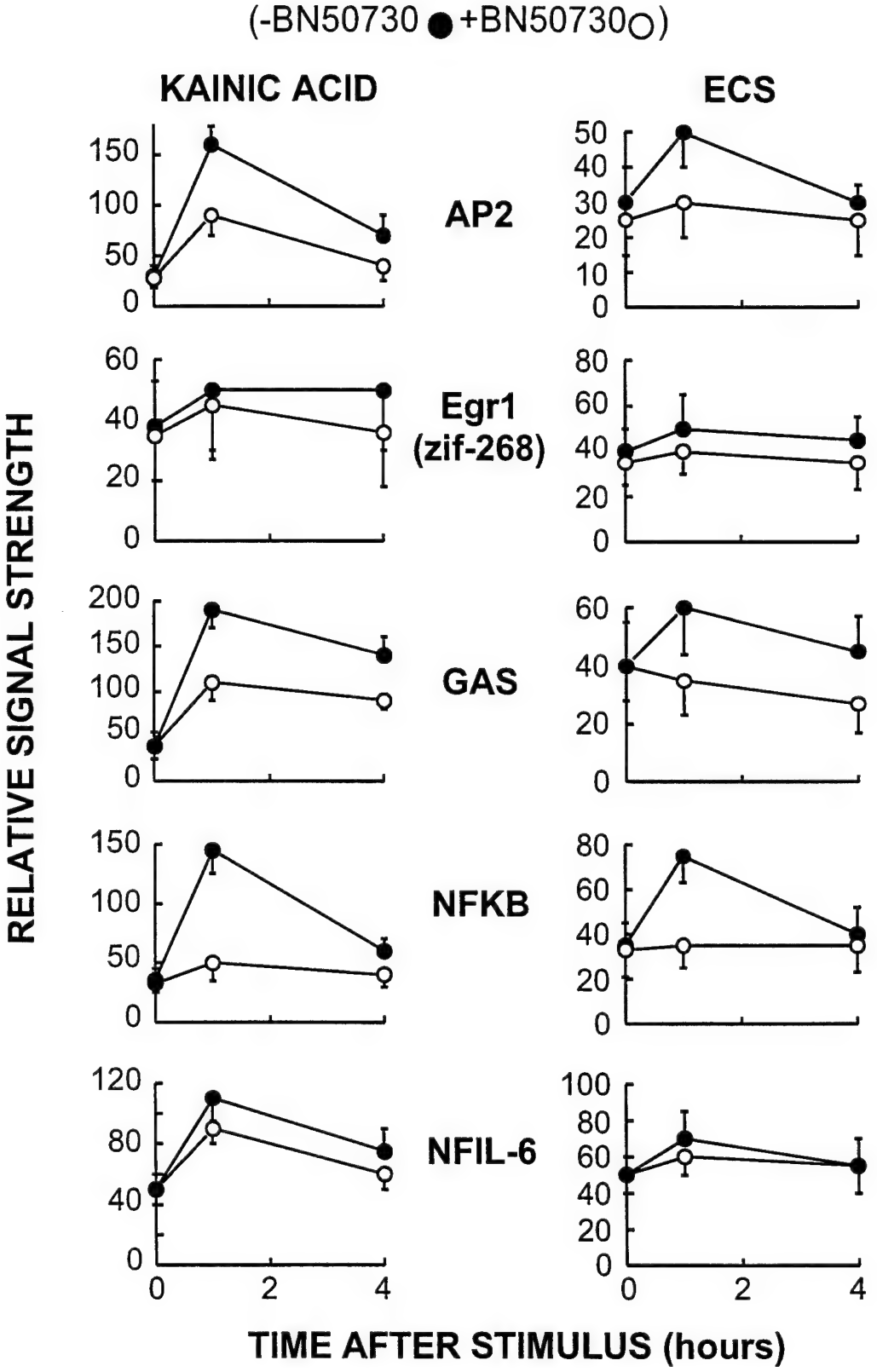
(-BN50730 ● +BN50730 ○)
KAINIC ACID
ECS
AP2
Egr1
(zif-268)
GAS
NFKB
NFIL-6
RELATIVE SIGNAL STRENGTH
TIME AFTER STIMULUS (hours)

FIGURE 6

Time course of induction of transcription factors AP2 and NFκB in PAF-treated IM9 cells onto the core consensus sequences 5'-GCCCGCGG-3' (AP2) and 5'-GGGGACTTTCCC-3' (NFκB), as determined by EMSA.

TIME COURSE OF BINDING OF TRANSCRIPTION FACTORS NFKB AND AP2 ONTO THEIR TARGET DNA SEQUENCES IN PAF-INDUCED IM9 CELLS

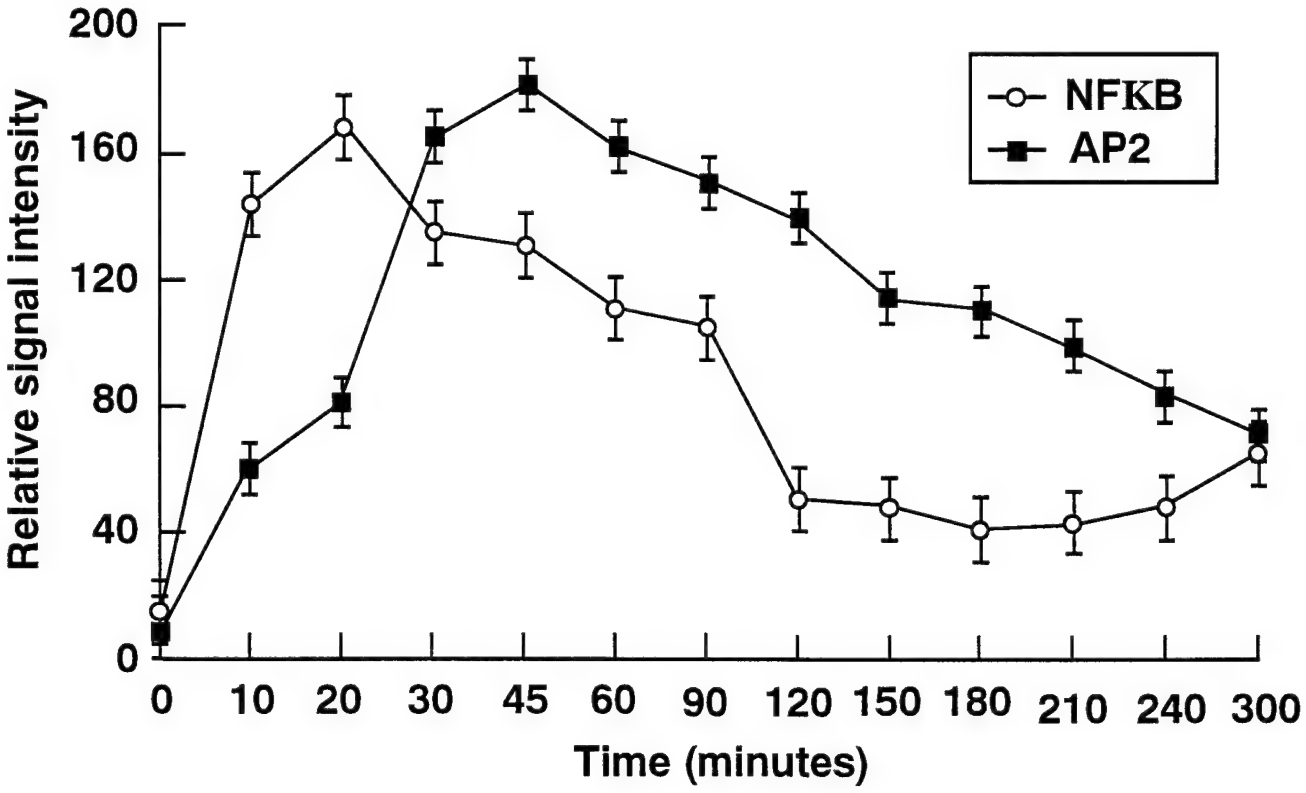

FIGURE 7

A release of inhibition of expression of the COX 2 gene may be mediated by repressor protein(s) located in the COX 2 promoter.

B. Nuclear protein extracts (NPXTs) were prepared from control and PAF-induced human IM9 cells and were incubated with single end-biotinylated DNA from -581 to -361 bp (top) and -296 to +35 bp and -454 to -410 bp (bottom) of the human COX 2 promoter. After utilizing a magnetic bead capture technique (DYNAL^{R}; 15), one ~32-40 kDa protein (arrow) appeared disappeared following PAF-mediated COX 2 gene induction. This probably represents one non-histone chromatin (NHCP) protein which assists in the regulation of COX 2 gene expression by recognizing some feature of the mouse -454 to -410 bp COX 2 promoter sequence (FIGURE 1 and 2). M = Novex MultiMark low molecular weight protein standards; detection limit is 200 pg/mm^2 in a 0.75 mm thick, 5-20% gradient acrylamide (30:1) Tris-glycine-SDS gel system.

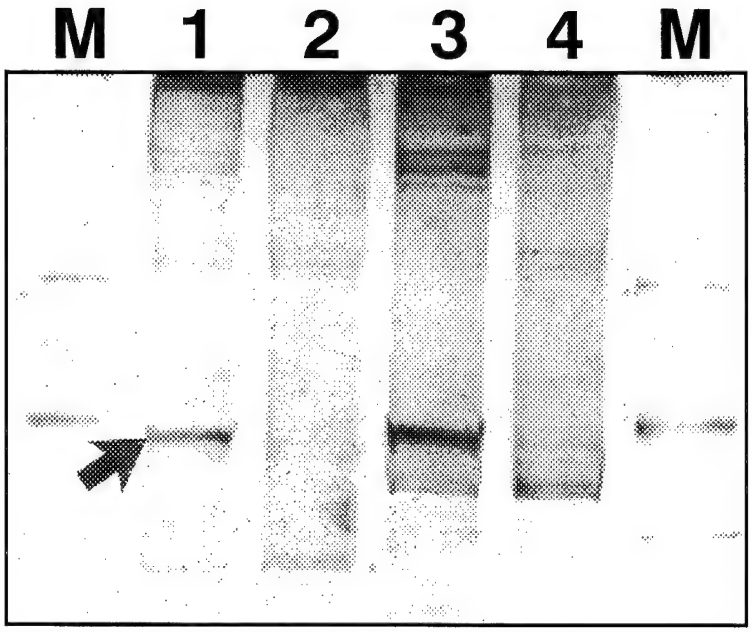
M
1
2
3
4
M

Figure 7
Bazan, Chapter 4, 1997

TABLE 1

TIS10/PGS2 promoter elements: species differences

Nine factors/elements identified in the proximal 5' region (-1000 to +1 bp) of the TIS10/PGS2 gene in mouse, rat and human which may be important in the induction and expression of this gene. Kodak/IBI DNA Sequence Analysis (Ver. 2.04) and EMSA (electrophoretic mobility shift assay)

FACTOR/ELEMENT	DESCRIPTION	NUMBER		
		Mouse	Rat	Human
$[A]_6$	$[A]_6$ kinks DNA helix 18°	14	13	10
AP1	activator protein 1	2	0	0
AP2	activator protein 2	1	2	1 or 2
cAMP	conserved CRE (cyclic AMP response element)	1	0	1
CpG	demethylation of CpG islands	10	8	6
NF-IL6	interleukin-6 type elements	3	4	6
NFκB	nuclear factor kappa B	3	4	3
SRE	serum response element	0	0	2
TATA/TFIID	TATA box and transcription factor IID	0	0	1

NEUROCHEMICAL PROTECTION OF THE BRAIN, NEURAL PLASTICITY AND REPAIR.

Platelet-activating factor as a presynaptic mediator of excitatory neurotransmitter release.

INTRODUCTION

Overstimulation of glutamate neurotransmission can lead to excitotoxic neuronal damage (Olney, 1986), and has been implicated in several degenerative diseases in the CNS (Choi, 1988). Its neurotoxicity is attributed to Ca^{2+} influx into postsynaptic neurons, mediated by the *N*-methyl-D-aspartate (NMDA) class of glutamate receptors (Rothman and Olney, 1986, 1987; Choi, 1988). Overstimulation of NMDA receptors therefore results in the overactivation of a whole range of calcium dependent pathways and the disruption of normal cellular functions. This laboratory has been interested for a long time in the neurotrauma-induced release of free fatty acids, including arachidonic acid (AA) and bioactive lipids such as platelet-activating factor (PAF, 1-*O*-alkyl-*sn*-glycero-3-phosphocholine), which results from the Ca^{2+}-mediated activation of phospholipase A_2 (PLA_2).

PAF has significant roles in neuronal physiology and pathophysiology. PAF is a stimulator of neuronal gene expression, acting through an intracellular receptor (Marcheselli and Bazan, 1994, 1996) properties which are considered elsewhere in this report. In this section, we are interested in the ability of PAF to modulate excitatory neurotransmission via a presynaptic, cell-surface receptor. PAF has been shown to stimulate glutamate release from hippocampal neurons in culture (Clark et al, 1992), and to involved in the development of glutamate-mediated long-term potentiation in the hippocampus (Del Cerro et al, 1990; Arai and Lynch, 1992; Wieraszko et al, 1993) through a presynaptic mechanism (Kato et al, 1994). Thus, under conditions of glutamate receptor overstimulation, the resulting accumulation of PAF could, in turn exacerbate presynaptic glutamate release and thus enhance excitotoxicity.

Membrane unsaturated fatty acid turnover and the synthesis of bioactive lipids are modulated by phospholipases A_2 (PLA_2), ubiquitous enzymes that catalyze the hydrolysis of *sn*-2-acyl ester bonds of phospholipids (PLs). There are several forms of PLA_2, including secretory

and intracellular enzymes. The secretory PLA_2 ($sPLA_2$) are relatively small proteins (M_r ~14 kDa), are active at submillimolar concentrations of calcium and do not display selectivity for the degree of saturation of fatty acid residues at the *sn*-2-position of PLs (Dennis, 1994; Kudo et al, 1993). Different families of $sPLA_2$s are found in pancreatic secretions (type I), platelets, neurons, mast cells, snake venoms, inflammatory exudates (type II), and bee venom (type III) (Dennis, 1994, Kudo et al, 1993; Davidson and Dennis, 1990). The major intracellular PLA_2 is the calcium-dependent $cPLA_2$ (type IV), which in contrast to the $sPLA_2$s has a higher molecular mass (85 kDa), is active at submicromolar Ca^{2+} concentrations, and shows selectivity for *sn*-2-arachidonoyl-PLs (Kudo et al, 1993; Fujimori et al, 1992). Within the brain, $cPLA_2$ is especially highly expressed in astrocytes (Stephenson et al, 1994). Secretory PLA_2 are also present in the brain. The expression of $sPLA_2$ type II is stimulated in the rat brain by ischemia/reperfusion (Lauritzen et al, 1994) and in cultured astrocytes by inflammatory mediators (Oka and Arita, 1991). $sPLA_2$ causes activation of Glu release in the rat cerebral cortex (O'Regan et al, 1995). $sPLA_2$ bind to cell surface receptors, the N type and the M type (Lambeau et al, 1989, 1990, 1994, 1995) identified using $sPLA_2$ purified from snake and bee venoms as ligands. Neurotoxic $sPLA_2$ from Taipan snake venom, OS2, and from bee venom bind to the N-type receptor with high affinity (Lambeau et al, 1989, 1990). Other $sPLA_2$s such as OS1, also purified from Taipan snake venom, display higher enzymatic activity than the $sPLA_2$s OS2 and bee venom (2.7- and 7-fold higher, respectively) (Lambeau et al, 1989). Although OS1 binds with high affinity to M-type receptors (Lambeau et al, 1990, 1994, 1995), it does not bind to N-type receptors (Lambeau et al, 1989) and is therefore non-neurotoxic. $sPLA_2$ type II is stored in synaptic vesicles and released by depolarization or neurotransmitter stimulation, and its secretion is coupled with the activation of catecholamine release (Matsuzawa et al, 1996). It is this co-release of $sPLA_2$ and neurotransmitters that has lead us to investigate the possible interrelationship between glutamate neurotransmission and $sPLA_2$ activity in excitotoxicity.

PAF is inactivated by removal of the acetate from the *sn-2* carbon, catalyzed by PAF acetylhydrolase (PAF-AH). PAF-AH are essentially $sPLA_2$ with a substrate specificity for short-chain fatty acyl groups (Tjoelker et al, 1995b). There are intracellular and secreted forms of PAF-AH. The major intracellular form in brain (Type II) is a complex enzyme, consisting of two different types of catalytic subunit and a regulatory subunit (Hattori et al, 1994a, b). The gene for the regulatory subunit is deleted in lissencephaly (Hattori et al, 1994b), a disorder of neuronal migration in the cerebral cortex. The major portion of secreted PAF-AH is associated with plasma lipoproteins (Stafforini et al, 1985), but an intracellular homologue (type Ib) has recently been cloned (Hattori et al, 1996). Both forms exist as ~40kDa monomer. Recombinant plasma PAF-AH (rPAF-AH) has been shown to be effective when administered in animal models of inflammatory disorders known to involve PAF (Tjoekler et al, 1995a). In this present study, we were interested in assessing the effects of exogenously-administered rPAF-AH on excitoxicity with the aims of confirming the role of PAF, and assessing the potential use of rPAF-AH as a pharmacological agent.

BODY

Previous work

Primary cultures of cortical and hippocampal neurons were established and the sensitivity to excitatory amino acids, $sPLA_2$, and PAF characterized. Conditions for excitatory amino acid toxicity were defined and used to set-up conditions for the confocal imaging of calcium fluxes in individual living neurons. The toxic $sPLA_2$ surprisingly decreased basal Ca^{2+} levels, in contrast to the large increases elicited by glutamate. Primary hippocampal cultures were found to be more sensitive to neurotoxic conditions than cortical cultures. Acute treatment with PAF was found to induce an average increase in calcium in hippocampal neurons, and in some cells caused more calcium oscillations. Short-term pretreatments also appeared to potentiate subsequent neuronal responses to submaximal concentrations of glutamate. In contrast, overnight treatments with PAF desensitized neurons to submaximal glutamate calcium responses (year 2).

The studies of year 1 supported a role for PAF as transynaptic modulator of excitatory synaptic transmission, demonstrating that PAF can cross the synapse and enhance the frequency of miniature excitatory synaptic currents acting upon neighboring presynaptic neurons.

Objectives, year 4.

1. In our previous experiments looking at the neurotoxic effects of combined $sPLA_2$, we had used high concentrations (80μM) of glutamate. Our aims were to extend this previous work by modulating both $sPLA_2$ and glutamate at submaximal concentrations to investigate how the combination of the two might affect cortical neurons.

2. A sustained increase in internal calcium concentration $[Ca^{2+}]_i$ has been associated with neurotoxicity. Sub-toxic concentrations of glutamate (~1μM) elicit only a transient calcium flux. Our aims were to monitor $[Ca^{2+}]_i$ dynamics in cortical neurons using laser scanning confocal microscopy and to determine if $sPLA_2$ could alter the calcium flux elicited by sub-toxic glutamate concentrations.

3. Our previous studies had shown PAF antagonists to be neuroprotective *in vivo*, implying a role for PAF in neuronal damage. We hypothesized that PAF was involved in the mediation of excititoxic neuronal death, and therefore that either lowering PAF concentrations or blocking PAF activity would inhibit excitoxicity. We had available to us human recombinant PAF acetylhydrolase (rPAF-AH), a gift of ICOS Corporation (Bothell, WA), as described by Tjoelker at al, 1995a. Our aims were to determine if rPAF-AH could inhibit excitoxicity, as assessed by lactate dehydrogenase release, and block apoptosis, the mechanism by which excitotoxic amino acids induce neuronal death.

MATERIALS AND METHODS

Primary neuronal cultures.

Cortical neuronal cultures - These were established from 15-day-old rat embryos as described previously (DeCoster et al, 1992a; DeCoster and Yourick, 1994). Cells were plated onto poly-L-lysine-coated dishes at a density of 4×10^5 cells/well in 48-well plates. The cells were cultured in neuronal culture medium containing 10% fetal calf serum and 10% horse serum in a 5% CO_2 incubator at 37°C. Astrocyte proliferation was prevented by adding cytosine arabinoside (10^{-5} M) at day 4 after plating, left for 3 days, and then replaced with minimum essential medium containing 10% horse serum. Cells were used for experiments at days 14-21 after plating. The composition of the cultures remained at approximately 80% neurons, 20% glia, as previously reported (DeCoster et al, 1995).

Hippocampal neuronal cultures - These were prepared using modifications of previously established techniques (Clark et al, 1992). Culture plates were coated with Matrigel (Collaborative Research) and stored at 37°C until plating. Hippocampi were dissected aseptically from 1-3 day old rat pups, treated with a 0.1% papain solution for 20 minutes, then triturated

with a pasteur pipette. Single cells were isolated from tissue pieces and plated at a density of 500,000 viable cells/ml in minimal essential medium plus 10% fetal calf serum, 10% horse serum, and supplemented with insulin, human transferrin and selenium, and maintained in 37°C, 5% CO_2 incubators. After 24 hours, cultures were treated with 10^{-5} M cytosine arabinoside (AraC). 4 days after plating, AraC was removed and the cells re-fed with fresh plating medium, this time lacking any serum. All experiments were carried out cells at least 11 days after plating.

Neuronal toxicity assay.

Lactate dehydrogenase (LDH) release was used to quantitatively assess cell injury. LDH release was measured 20h after exposure to $sPLA_2$ and/or Glu. Cells were exposed for 45 min at room temperature to highly purified $sPLA_2$ from bee venom and snake venom (OS1 or OS2) and /or Glu (80 μM) in Locke's solution without magnesium. Locke's solution was exchanged for minimum essential medium without phenol red, and the cultures were returned to the incubator for 20h at 37°C. Previous studies had shown that this procedure results in minimal LDH release before 12h, and maximal release by 20h. LDH release was assayed using a commercially available kit (Sigma, St. Louis, MO) according to the manufacturer's instructions.

Apoptosis methods.

TUNEL staining:- Terminal deoxynucleotidyl transferase dUTP nuclear end labelling (TUNEL) staining was performed using the Apoptosis Detection System, Fluorescein (Promega, Madison, WI). Fixed cells were counter-stained with propidium iodide (Sigma, St Louis, MO). Digitized images were collected using fluorescence microscopy and a Hammamatsu chilled 3-CCD color camera. Fluorescein-positive cells were counted using Image Pro Plus (Media Cybernetics, Silver Spring, MD) two-dimensional graphics analysis software. The data were then expressed as a percentage of apoptotic cells compared to non-stained cells.

Cell death ELISA:- An ELISA assay for cell death, based on the detection of mono- and oligo-nucleosomes, was carried out using a commercially-available kit (Boehringer-Mannheim, Indianapolis, IN). Briefly, cells were homogenized in a phosphate buffer containing a cocktail of protease inhibitors (0.5 mM PMSF, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 2 μg/ml soy bean (soy bean). Protein content into the samples were normalized, then samples were diluted 10 fold with a lysis buffer provided by the ELISA kit. 20μl aliquots in a volume of were loaded in 96 well plates, and incubated for two hours with 80μl of incubation buffer containing the monoclonal antibodies directed against DNA and histones for detection of mono and oligo-nucleosomes. After washing the unreacted antibodies, the immunocomplex of DNA-histone-antibodies, remained bound to the streptavidin coated plates, and detected using a horseradish peroxidase-linked secondary antibody . Quantitative analysis was performed in a Spectramax-250 96-well plate reader (Molecular Devices).

DNA laddering:- Neuronal cellular DNA was extracted using the method of Tso et al, 1994 and electrophoresed on 1.8% agarose-TBE gels. Images of ethidium bromide-stained gels were captured using a Gel Doc 1000 video system with Molecular Analyst software (Bio Rad, Hercules, CA).

Electron microscopy:- Neuronal cells were prepared for electron microscopy using standard procedures (Gordon and Bazan, 1993) Briefly, cells were scraped from the surface of the culture dish and pelleted by brief centrifugation. Culture medium was removed and the cell pellet was fixed in 2% glutaraldehyde, 2% formaldehyde, 0.135M sodium cacodylate buffer (pH 7.3) overnight at 4°C. The pellet was then rinsed three times in cacodylate buffer and post-fixed for 1h in 1% OsO_4 in cacodylate buffer. After rinsing a further three times in cacodylate buffer, the pellet was run through an ethanol 20% step dehydration series to acetone. Infiltration and plastic embedding occurred in a mixture of Epon-araldite plastic. Silver-gold sections were placed on

parlodion-coated, 100 mesh, hexagonal nickel grids and contrasted with uranium and lead salts. Sections were viewed with a Zeiss C10 transmission electron microscope, and photographed on 3.25" x 4" Kodak electron microscope film 4489 (EMS, Ft Washington, PA).

Measurement of intracellular calcium

For measurements of calcium dynamics in cell cultures, cells were loaded with the fluorescent calcium indicator fluo-3 for one hour as described in DeCoster et al, 1992a. Excess dye was removed after the loading period and replaced with Locke's solution without magnesium and supplemented with 1μM glycine. After a recovery period of 30 minutes at 37°C, calcium dynamics were monitored with a Noran Instruments Odyssey XL confocal laser scanning microscope. Digitized images were captured on a Silicon Graphics Indy workstation, and calcium changes were analyzed using Noran Intervision software on Indy workstations. Additions of glutamate and/or $sPLA_2$ were made by adding an appropriate volume of the compound into the well by pipette. Controls were carried out by adding an appropriate volume of Locke's solution. Using this method, compounds remained in the culture well for the remainder of the scanning period. All calcium imaging experiments were conducted at room temperature. On every plate, experimental conditions were paired with controls, and conditions carried out on at least three separate platings of cells.

RESULTS

Effects of sub-toxic concentrations of $sPLA_2$ and glutamate on LDH release from cortical neurons.

Concentrations of bee venom $sPLA_2$ and glutamate, which alone are non-toxic, show significant and synergistic toxicity in combination. Neither 25μM glutamate nor 25ng/ml $sPLA_2$ are toxic to cortical neurons when added alone, but when added together elicit LDH release that is both significantly more than controls and more than additive (Figure 1A). Similar results were obtained using a concentration of $sPLA_2$ (50ng/ml) that was barely toxic, and varying the glutamate concentration between 10μM and 25μM (Figure 1B).

Effects of $sPLA_2$ on $[Ca^{2+}]_i$ fluxes elicited by sub-toxic concentrations of glutamate.

1μM glutamate, a sub-toxic concentration, elicits a transient $[Ca^{2+}]_i$ flux (Figure 2A). However, when a sub-toxic concentration of glutamate (25ng/ml) is added 3 minutes before glutamate, neurons respond to 1μM glutamate with a sustained $[Ca^{2+}]_i$ increase (Figure 2B). Decreasing the time between $sPLA_2$ and glutamate additions reduces the enhancement of the $[Ca^{2+}]_i$ response. When $sPLA_2$ is added 30 seconds before glutamate, only a partial enhancement of $[Ca^{2+}]_i$ is seen (Figure 2C).

$sPLA_2$ also enhances $[Ca^{2+}]_i$ fluxes elicited by a concentration of glutamate (250nM) that, by itself, stimulates $[Ca^{2+}]_i$ in only a small proportion of neurons (threshold concentration) (Figure 2A, B). Addition of 50ng/ml $sPLA_2$ before 250nM glutamate greatly increases the recovery time of cells to baseline (Figure 2C).

Figures 2 and 3 show data from representative neurons. A quantitative summarization of the data are presented in Table 1.

Neuroprotective effects of rPAF-AH.

Recombinant human PAF-AH (rPAF-AH) exhibited neuroprotection against N-methyl-D-aspartate (NMDA)-mediated neurotoxicity in hippocampal neurons. rPAF-AH (20μg/ml final concentration) was added to cultures 30 minutes before NMDA (50μM). The medium was changed 120 minutes later and replaced with fresh medium without NMDA, but containing 10μg/ml rPAF-AH. The cultures were then incubated for a further 22 hours prior to analysis of LDH release, TUNEL staining, DNA laddering, or electron microscopy.

As assessed by LDH release, rPAF-AH gave significant protection against NMDA-induced neurotoxicity (Figure 4). TUNEL staining revealed less stained nuclei in cultures pre-treated with rPAF-AH (Figure 5) than in cultures treated with NMDA alone (Figure 6), but more than untreated controls (Figure 7). Increasing the time of preincubation with rPAF-AH before addition of NMDA slightly increased the neuroprotection (Figure 8). The ELISA assay for mono- and oligo-nucleosome release also showed decreased apoptosis in cells pretreated with rPAF-AH (Figure 9). None of the assays showed complete neuroprotection, as was confirmed by DNA laddering, which showed significant DNA fragmentation even in the rPAF-AH treated cells (Figure 10). Electron microscopy of cultures showed nuclei in NMDA-treated cells characteristic of cells undergoing apoptosis (Figure 11), with shrunken nuclei and highly-condensed chromatin. By contrast, the nuclei of rPAF-AH/NMDA-treated cells (Figure 12) looked very similar to those of untreated cells (Figure 13).

CONCLUSIONS

1. Concentrations of bee venom $sPLA_2$ (20ng/ml) or glutamate (25μM) which are not toxic by themselves can, in combination, produce toxicity in cortical neuron cultures, and a sub-toxic concentration of glutamate (10μM) produces synergistic toxicity combined with a marginally toxic concentration of $sPLA_2$ (50ng/ml).

2. The ability to enhance glutamate-induced $[Ca^{2+}]_i$ fluxes in cortical neurons may provide a mechanism by which $sPLA_2$ stimulates excitotoxicity. Shorter preincubations with $sPLA_2$ reduce the enhancement of $[Ca^{2+}]_i$ fluxes, suggestive of a finite time required for the accumulation of PLA_2 metabolites of membrane lipids and/or activation of a signaling pathway(s) mediated through the N-type $sPLA_2$ receptor. Neuronal excitoxicity may therefore involve not just the activation of glutamate receptors, but may also include the action of $sPLA_2$ co-released with neurotransmitters and enhancing calcium fluxes, possibly through NMDA receptors.

3. rPAF-AH partially inhibits NMDA-mediated cell death in hippocampal neuron cultures. The reduction in toxicity, as assessed by LDH release, correlates with a reduction in apoptosis, as measured by TUNEL staining, release of nucleosomes, and nuclear morphology at the EM level. This suggests that PAF has a role in neuronal apoptosis induced by activation of the NMDA receptor. rPAF-AH could exert its effects by inactivating the PAF produced as a result of the NMDA/Ca^{2+}- mediated activation of $cPLA_2$, and which diffuses to the presynaptic termini to stimulate further glutamate release. It is not known whether rPAF-AH is taken up by neurons and could thus hydrolyze intracellular PAF in the post-synaptic neurons.

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TABLE 1.

Enhancement of neuronal glutamate-stimulated $[Ca^{2+}]_i$ by $sPLA_2$

[Glutamate]	[$sPLA_2$]	Mean recovery time	Mean % recovery	n
250nM	0	15.1±8.6 secs	103.0±2.2	16
250nM	50ng/ml	128.5±8.1 secs	104.6±3.6	18
1μM	0	>84 secs	66.1±9.7	8
1μM	25ng/ml	>84 secs	5.1±1.4	14
1μM	25ng/ml (30 secs)	>84 secs	70.5±11.8	7

FIGURE 1

Co-addition of $sPLA_2$ from bee venom plus glutamate results in synergistic toxicity as measured by total LDH release from cortical neuronal cultures.

A: under conditions where neither glutamate (25 μM) nor $sPLA_2$ (25 ng/ml) were significantly toxic when added alone, the combination of both agonists together resulted in significant and synergistic toxicity.

B: addition of 10 μM glutamate alone is not toxic, while 50 ng/ml of $sPLA_2$ is slightly toxic. When the two are added together, the toxicity is more than additive. Similar results were obtained when combining 25 μM glutamate and 50 ng/ml of $sPLA_2$. All data shown are the averages of at least triplicate wells with standard error of the mean shown. Dotted horizontal line indicates the LDH release from control wells, which has been normalized to 100%.

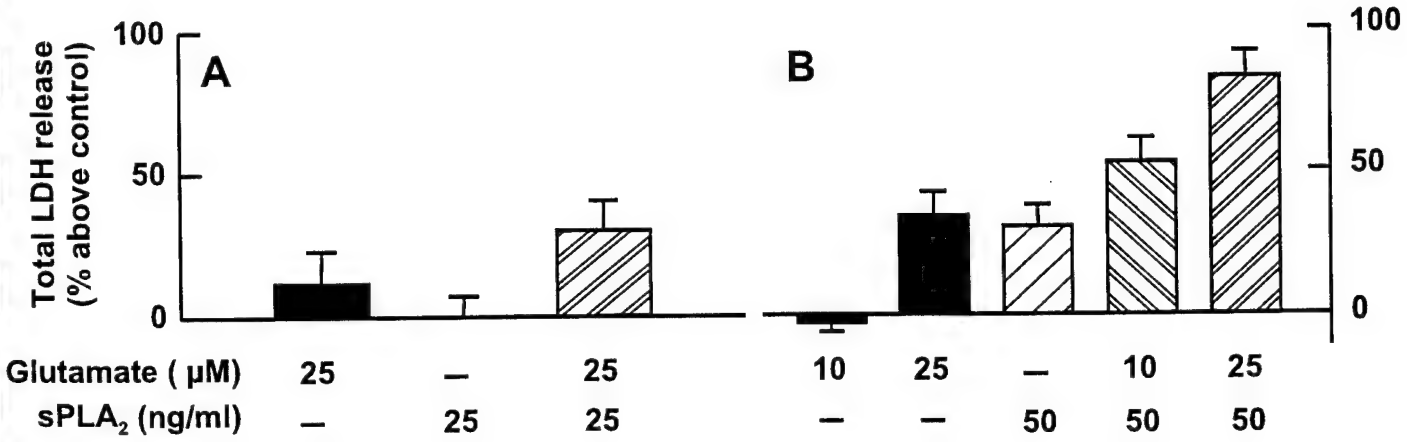

Total LDH release
(% above control)
100
50
0
A
B
100
50
0
Glutamate (μM) 25 — 25 10 25 — 10 25
sPLA2 (ng/ml) — 25 25 — — 50 50 50

FIGURE 2

Addition of $sPLA_2$ from bee venom greatly enhances the subsequent calcium flux in cortical neurons exposed to 1 μM glutamate.

A: addition of 1 μM glutamate alone to cortical neurons results in a transient change in [Ca2+]i, with cells quickly returning to baseline levels.

B: In comparison, addition of $sPLA_2$ minutes prior to 1 μM glutamate exposure results in neurons responding to glutamate with sustained $[Ca^{2+}]_i$. Data in each panel represent tracings from 3 representative neurons. $[Ca^{2+}]_i$ has been normalized to the time zero values for each individual cell.

C: Short pretreatment with $sPLA_2$ only partially enhances calcium flux in cortical neurons. Cortical neurons were exposed to $sPLA_2$ for only 30 seconds prior to glutamate addition. While one representative cell (circles) shows a sustained $[Ca^{2+}]_i$ increase, other cells (squares and triangles) quickly return to baseline levels. However, note that these latter two cells appear to spontaneously start to increase in $[Ca^{2+}]_i$ towards the end of the scan, without additional glutamate exposure.

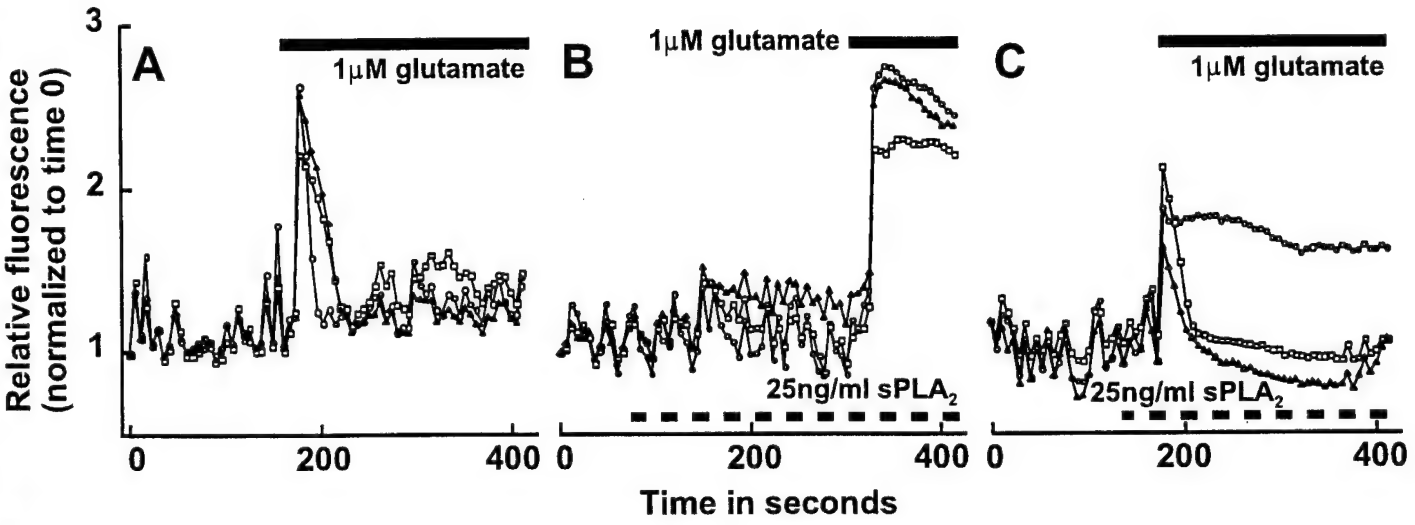

Relative fluorescence
(normalized to time 0)
3
2
1
A
1μM glutamate
B
1μM glutamate
25ng/ml sPLA2
C
1μM glutamate
25ng/ml sPLA2
0
200
400
Time in seconds

FIGURE 3.

Addition of $sPLA_2$ from bee venom greatly enhances calcium flux in cortical neurons exposed to threshold concentrations of glutamate.

A: Addition of 250 nM glutamate to cortical neurons results in no increase in $[Ca^{2+}]_i$, while 500 nM elicits a transient response.

B: In other cells, 250 nM glutamate addition causes a single (threshold) $[Ca^{2+}]_i$ event, with neurons returning to baseline within one scan. Note that in both cases, addition of Locke's solution causes no increase in $[Ca^{2+}]_i$.

C: Pretreatment of neurons with 50 ng/ml of $sPLA_2$ greatly increases the recovery time of cells to baseline.

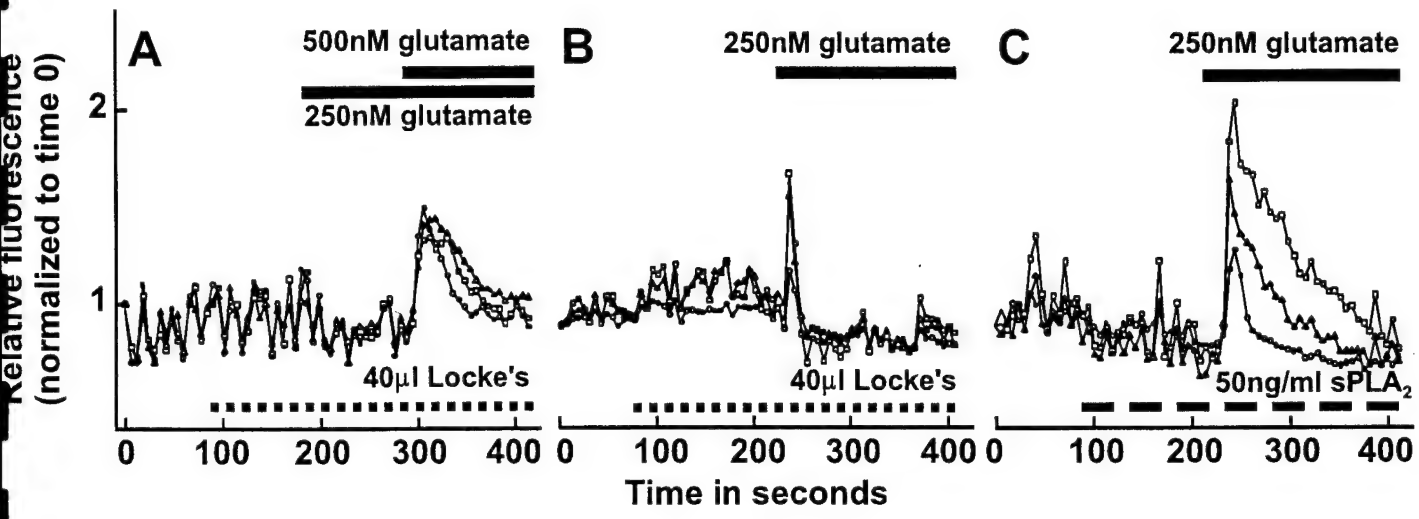

A
500nM glutamate
250nM glutamate
40µl Locke's
B
250nM glutamate
40µl Locke's
C
250nM glutamate
50ng/ml sPLA2
Relative fluorescence
(normalized to time 0)
2
1
0
100
200
300
400
Time in seconds

FIGURE 4

Effect of rPAF-AH on NMDA-induced neurotoxicity in hippocampal neurons as assessed by LDH release.

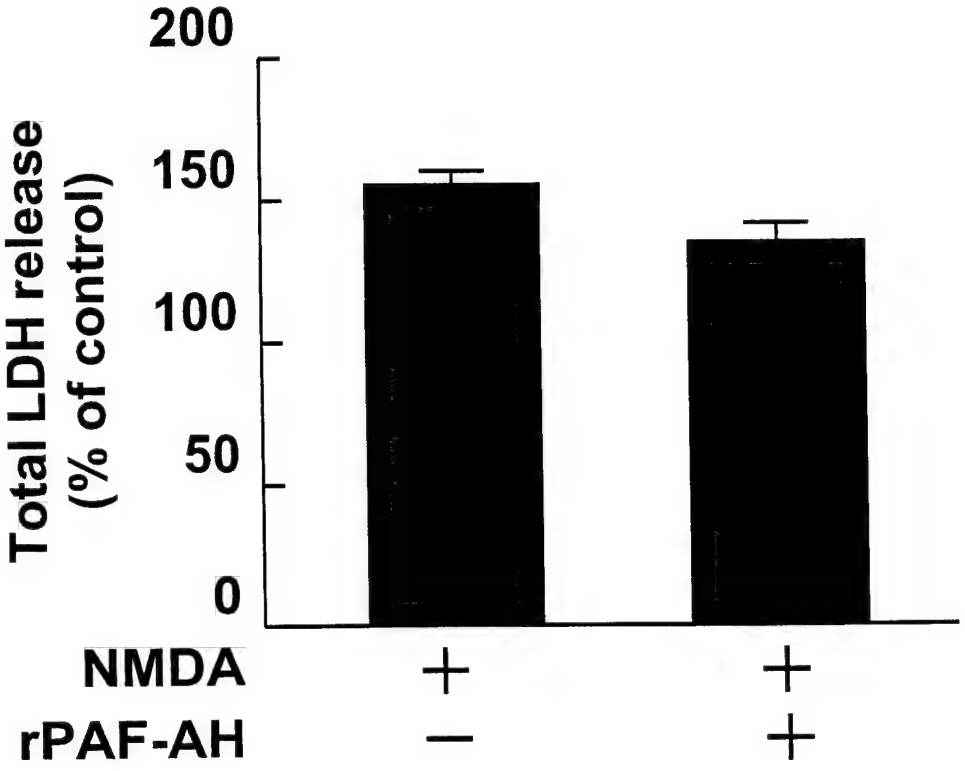

200
150
100
50
0
Total LDH release
(% of control)
NMDA
rPAF-AH
+
+
—
+

FIGURE 5.

Fluorescence microscopy of hippocampal neurons treated with rPAF-AH and NMDA, and TUNEL stained 24 hours after NMDA treatment. Apoptotic nuclei stain green (FITC-12-dUTP) while total nuclei stain red (propidium iodide).

Original magnification 200x

NMDA + PAF-AH

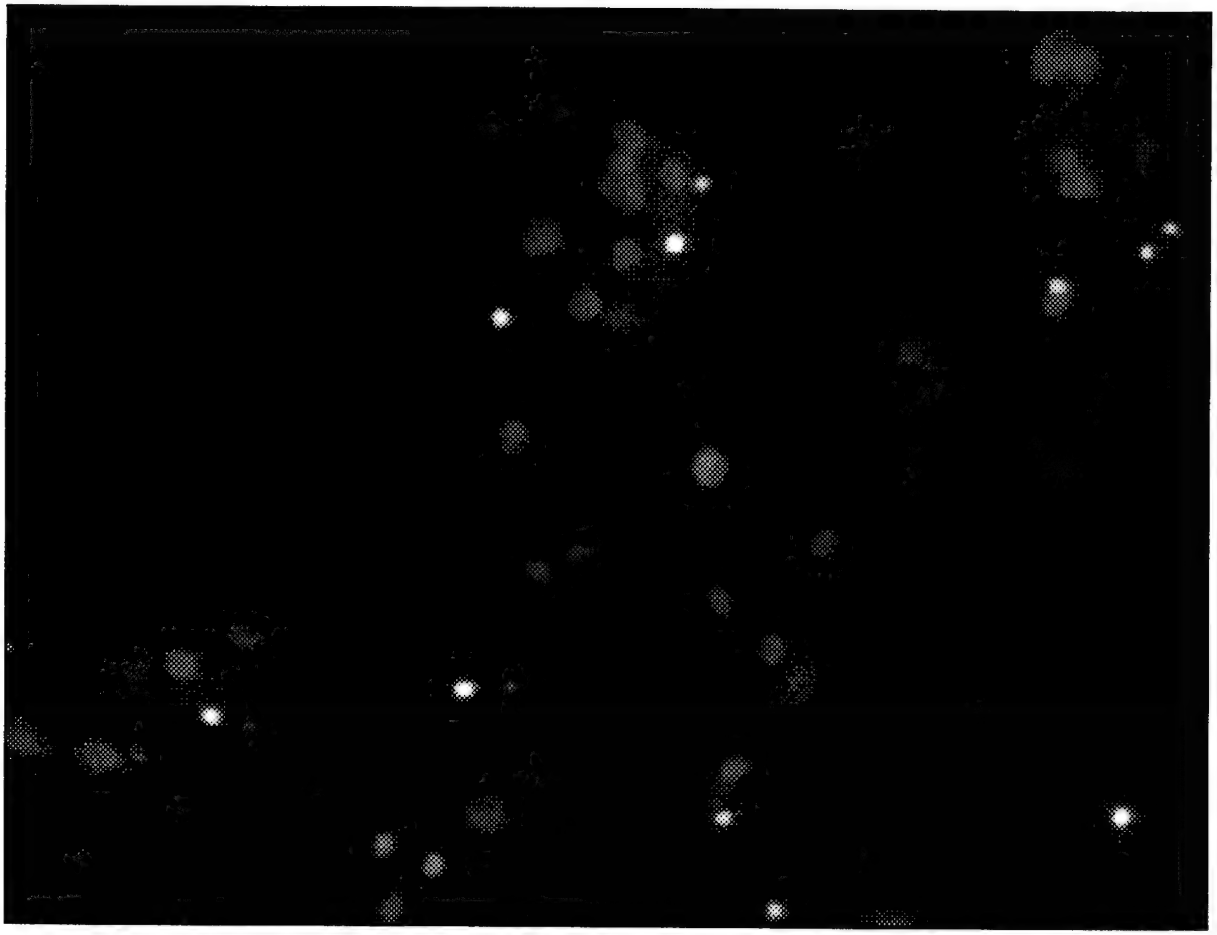


FIGURE 6.

Fluorescence microscopy of hippocampal neurons treated with NMDA, and TUNEL stained 24 hours after treatment.

Original magnification 200x

NMDA-treated cells

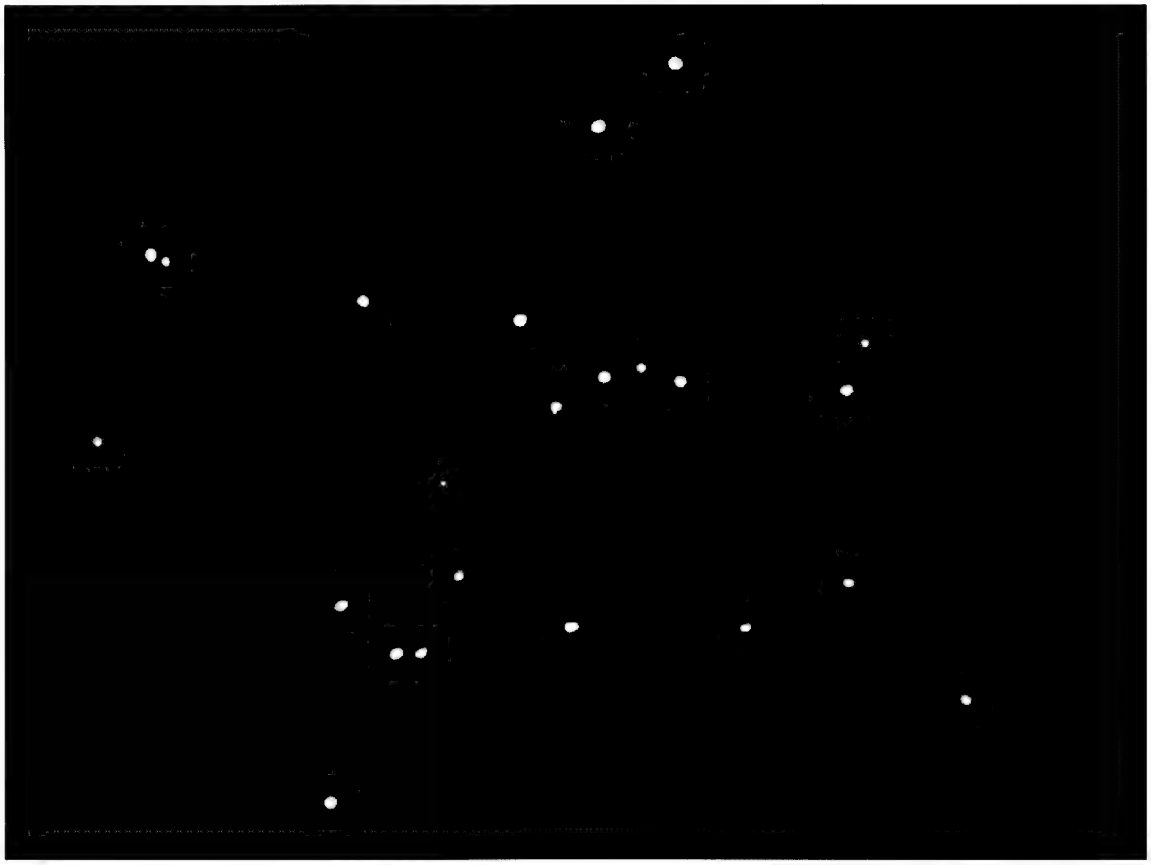


FIGURE 7.

Control hippocampal neuron cultures double-stained with TUNEL and propidium iodide. Original magnification 200x

Control cells

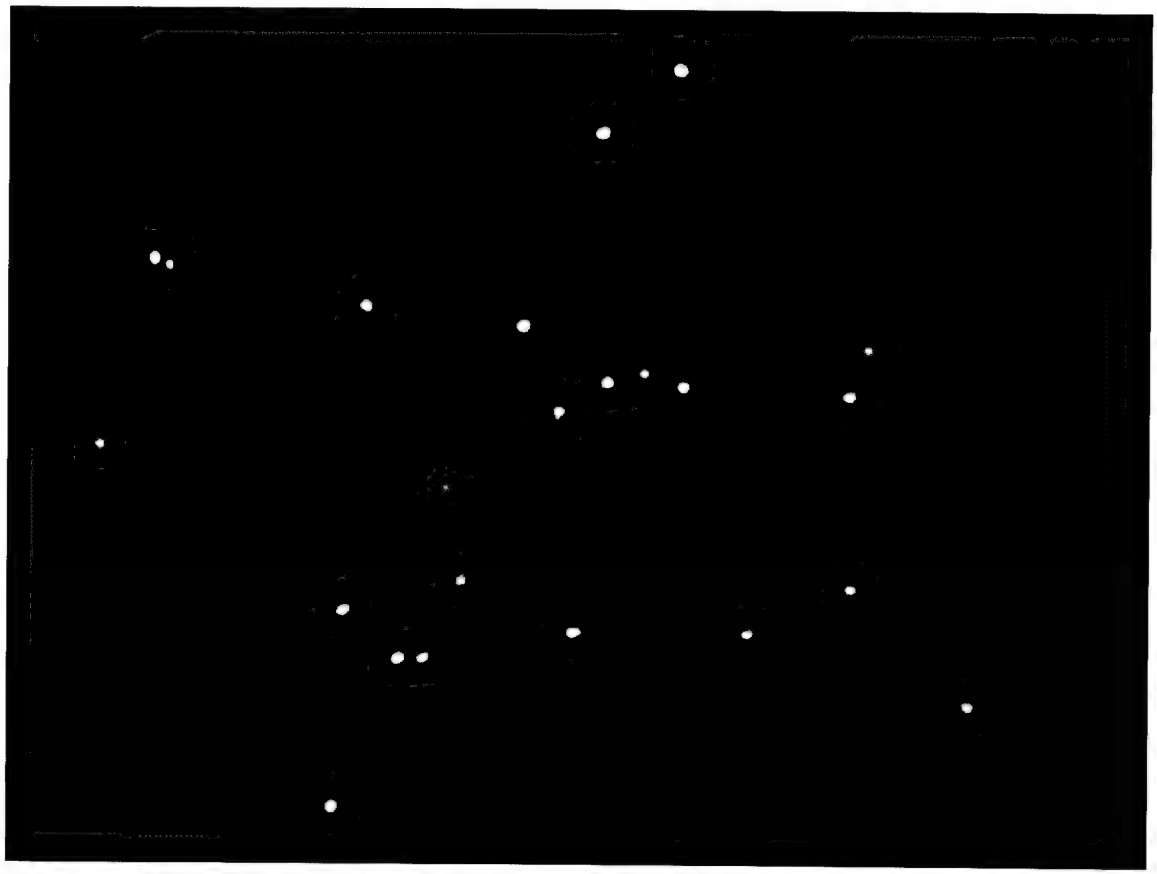


FIGURE 8.

Neuroprotection by rPAF-AH on NMDA-induced apoptosis, quantification of TUNEL-stained nuclei. The time course represents the time before NMDA addition at which the initial addition of NMDA was made. rPAF-AH (20ng/ml final) was added at defined times before addition of NMDA (50μM). The medium was replaced 120 minutes with medium containing no NMDA and 10ng/ml rPAF-AH.The cells were processed for TUNEL 22 hours later.
The data are from a representattive experiment with measurements made in at least triplicate, and are expressed as mean ± standard deviation.

rPAF-AH PROTECTS NEURONS IN CULTURE FROM GLUTAMATE RECEPTOR AGONISTS-INDUCED APOPTOSIS

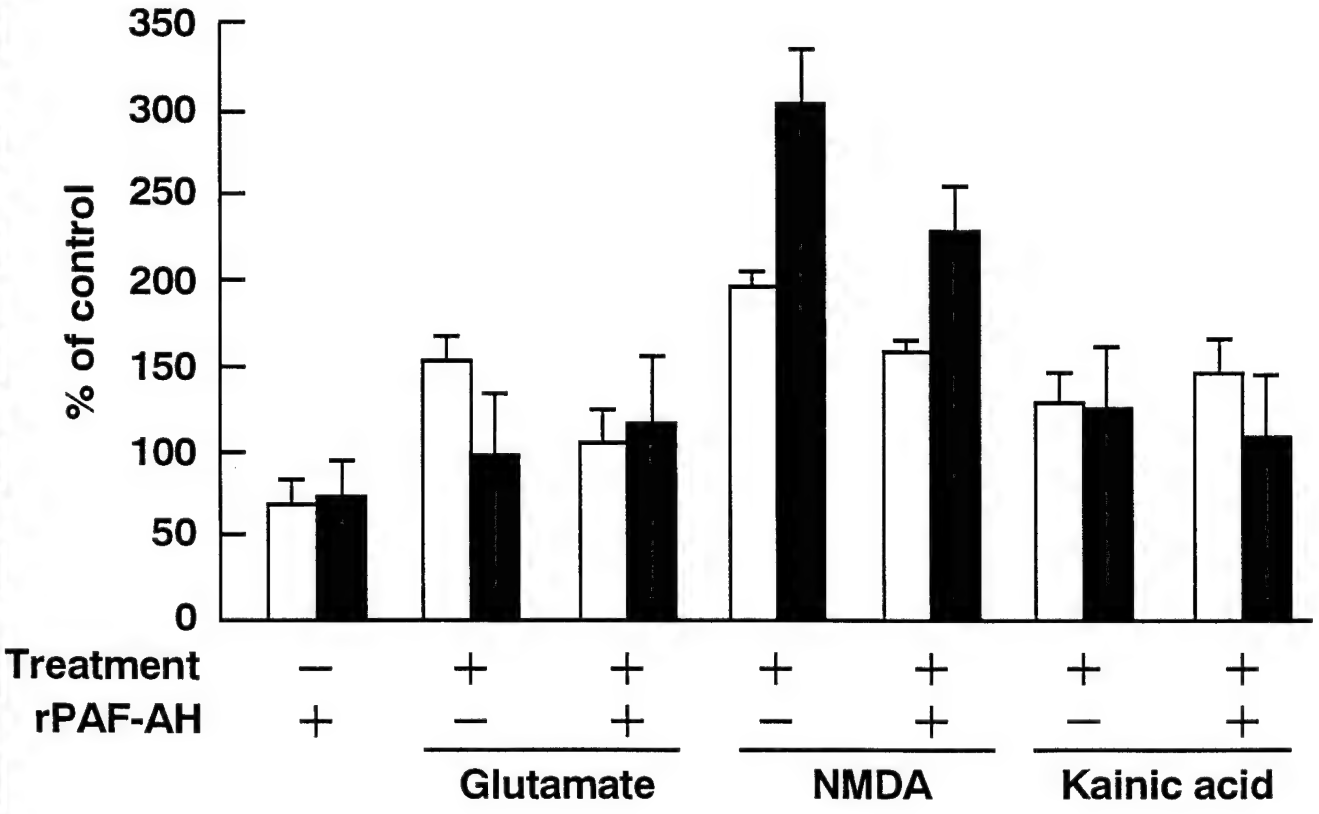


FIGURE 9.

Inhibition of NMDA-induced apoptosis in hippocampal neurons by rPAF-AH. Cultures were treated as for the LDH and TUNEL studies, and then assayed for mono- and oligo-nucleosomes using a commercially-available ELISA kit (Boehringer-Mannheim, Indianapolis, IN).

Data expressed as mean ± standard deviation (n=6)

rPAF-AH DECREASES APOPTOSIS IN NMDA-TREATED HIPPOCAMPAL NEURONS

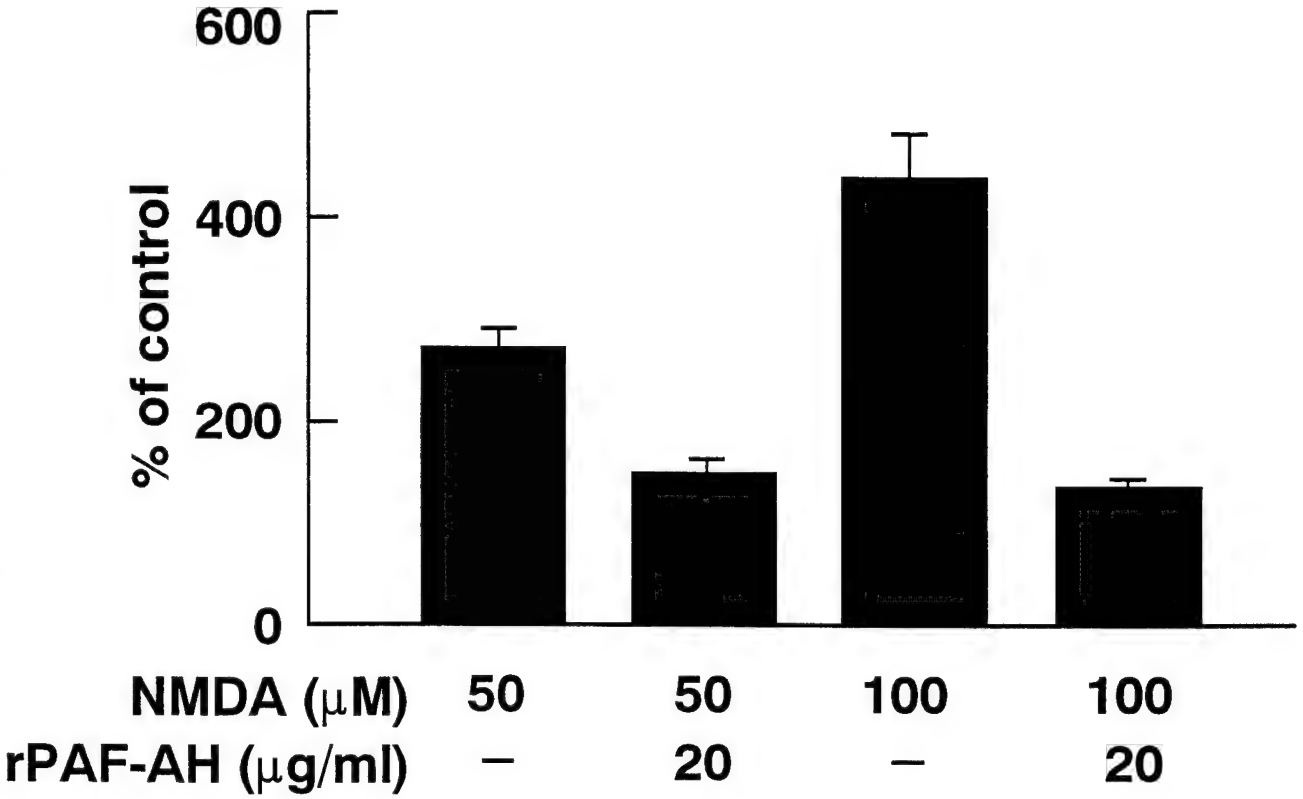


FIGURE 10.

Apoptotic DNA laddering induced in hippocampal neurons by NMDA treatment. 1.8% agarose/TBE gel of cellular DNA stained with ethidium bromide.

100bp ladder - DNA marker ladder of fragements in increments of 100 bp

C - Control (untreated) cultures.

N+P - Hippocampal neurons treated with NMDA and rPAF-AH.

N - Hippocampal neurons treated with NMDA alone.

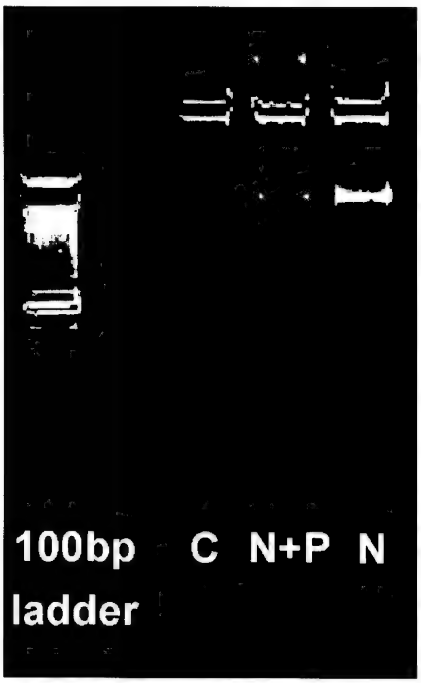

100bp
ladder
C
N+P
N

FIGURE 11.

Electron micrograph of hippocampal neurons treated with 50μM NMDA under the same conditions as other experiments in this series. Note that compared with cells treated with rPAF-AH + NMDA (Figure 12) and control cultures (Figure 13) the nucleus is highly contracted with condensed, densely-staining chromatin.

Original magnification 4000x

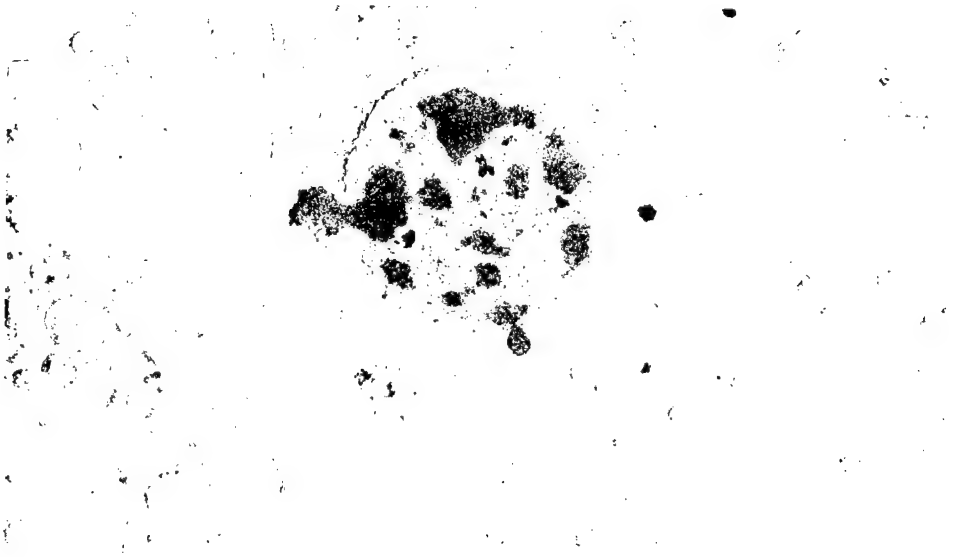


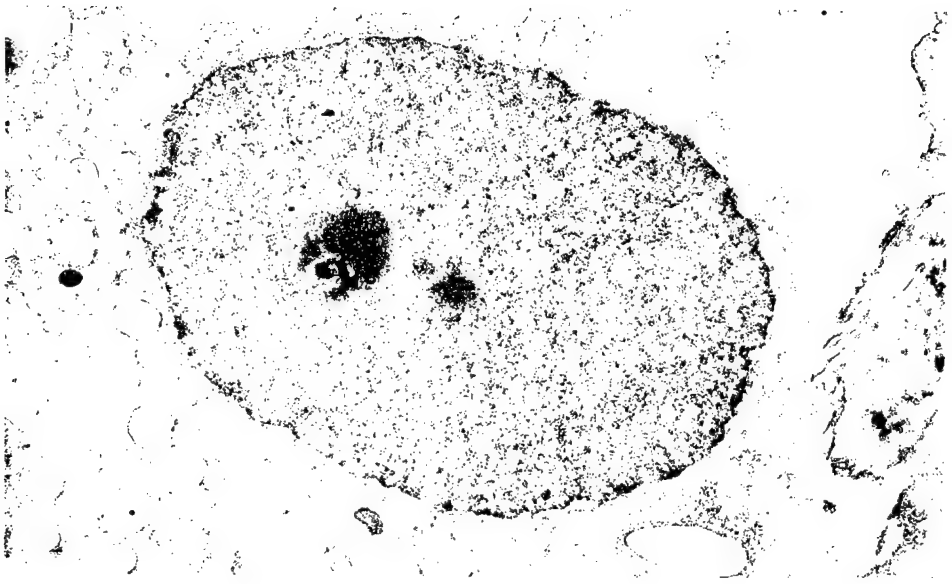
FIGURE 12.

Electron micrograph of hippocampal neurons treated with rPAF-AH and NMDA under the same conditions as other experiments in this series. Note that the nuclear morphology is essentially the same as control (untreated) cultures (Figure 13).

FIGURE 13.

Electron micrograph of hippocampal neurons from untreated cultures.

Original magnification 4000x



Addendum

1. DeCoster MA, Mukherjee PK, and Bazan NG. Platelet-activating factor is a downstream messenger of kainate-induced activation of MAP kinases in primary hippocampal neurons. *Journal of Neuroscience Research*, 1998 (in press).

2. Ogden, F, DeCoster, MA , and Bazan, NG. Recombinant plasma-type platelet-activating factor acetylhydrolase attenuates NMDA-induced hippocampal neuronal apoptosis. *Journal of Neuroscience Research*, 1998 (in press).

3. Bazan, NG. Bioactive lipids and gene expression in neuronal plasticity. *Advances in Experimental Medicine and Biology*. Plenum Publishing Co., 1998 (in press).

4. Bazan, NG, and Serou, MJ. Second messengers, long-term potentiation, gene expression and epileptogenesis. *Basic Mechanisms of the Epilepsies*. Lippincott-Raven Publishers, 1998 (in press).

5. Bazan NG. The neuromessenger platelet-activating factor in plasticity and neurodegeneration. *Progress in Brain Research*. Elsevier Science Publishers: Academic Publishing Division, 1998 (in press).

Platelet-activating factor is a downstream messenger of kainate-induced activation of MAP kinases in primary hippocampal neurons.

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Running title: PAF and kainate activate MAP kinases in neurons

ABSTRACT:

Excitatory amino acids (EAAs) transduce physiological and pathological signals to neurons. Similarly, the neuroactive lipid platelet-activating factor (PAF) has been implicated in modulating long-term potentiation (LTP) and neuronal survival. EAAs and PAF have been shown to increase mitogen-activated protein kinases (MAPKs) in different cell types. Here, we have investigated the similarities and differences between PAF and kainate in activating MAPKs in primary hippocampal neurons *in vitro*. ERK (extracellular signal-regulated kinase), JNK (c-JUN NH_2), and p38 kinases were activated by kainate or PAF in hippocampal neurons. This activation was blocked by the receptor antagonists CNQX and BN 50730 for kainate and PAF, respectively. The PAF receptor antagonist BN 50730 also blocked kainate activation. CNQX had no effect on PAF activation of the kinases, indicating that PAF is downstream of kainate activation. Co-application of submaximal concentrations of PAF and kainate resulted in a less than additive activation, suggesting similar routes of activation by the two agonists. Both CNQX and BN 50730 blocked kainate-induced neurotoxicity. These results indicate that PAF and kainate activate similar kinase pathways. Therefore, PAF acts downstream of the kainate subtype of glutamate receptors, and, when excessive receptor activation takes place, this bioactive lipid may contribute to neuronal cell death.

INTRODUCTION:

The MAP kinase cascade is activated in response to signals as diverse as growth factors and stimuli leading to apoptosis. Members of the MAP kinase family include ERK, JNK, and p-38 subtypes. In studies using PC-12 cells and nerve growth factor (NGF) withdrawal, Xia et al. (1995) proposed that the balance between the growth factor-activated ERK and stress-activated JNK-p38 kinase pathway may determine cell survival. However, studies using primary sympathetic neurons that die when deprived of NGF demonstrated that neuronal survival in these cells is independent of MAP kinase activation (Creedon, et al., 1996).

A wide range of signals are transduced by EAAs, resulting in physiological modulation such as modulation of synaptic function (Kleinschmidt et al., 1987; Fox et al., 1991; Madison et al., 1991; Rabacchi et al., 1992). Glutamate is the major mammalian brain EAA neurotransmitter, and it acts through the ionotropic and the metabotropic receptors (Nakanishi, 1992; Seeburg, 1993). The ionotropic subtype of glutamate receptors includes NMDA and AMPA/kainate receptors. Glutamate is also important in neurotoxicity, as demonstrated *in vitro* (Choi et al., 1987), and has been implicated in brain injury after stroke (Zivin and Choi, 1991). Activation of the AMPA/kainate receptor using the glutamate analog kainate, causes neurotoxicity in cell culture (Koh et al., 1990).

In the brain, stimulation elicited by ischemia or seizures leads to the accumulation of PAF (Kumar et al., 1988). This potent neuroactive lipid has been implicated in modulating glutamate release (Clark et al., 1992) and long-term potentiation (LTP) (Kato et al., 1994). PAF itself has

been shown to exert neurotoxic action in cell lines (Kornecki et al., 1988) and primary neuronal cultures (Nogami et al., 1997), although high concentrations of PAF were required in both studies to elicit toxicity.

Both PAF and glutamate have been shown to activate the MAP kinases. Honda et al. (1994) demonstrated that PAF receptors transfected into Chinese hamster ovary cells could activate MAP kinase. The addition of PAF to human neurtrophils stimulated the activity of p42erk2 MAP kinase (Fouda, et al., 1995), and recently, PAF-induced mitogenesis in an endometrial adenocarcinoma cell line has been postulated to involve the activation of MAP kinase (Bonaccorsi, et al., 1997). Application of glutamate to primary cultured neurons from both hippocampus and cortex has been shown to activate MAP kinases (Bading and Greenberg, 1991; Fiore, et al., 1993). Work by Kurino et al. (1995) reported that MAP kinase was activated by stimulation of glutamate receptors in cultured rat hippocampal neurons. These experiments indicated that stimulation of MAP kinase occurred through NMDA receptors, but not through non-NMDA receptors.

Here we report that ERK, JNK, and p38 kinases are all activated by application of PAF or kainate to primary hippocampal neurons. Using PAF- and kainate-receptor antagonists, we demonstrate that kainate and PAF utilize a common route for activating the MAP kinases. Since application of kainate to hippocampal neurons was neurotoxic at the concentrations used here to elicit kinase activation, but application of PAF was not toxic, common and multiple pathways of kinase activation may be involved in conditions of neuropathology and neuronal plasticity.

MATERIALS AND METHODS:

Materials and Antibodies - PAF, KA, Glutathione S- transferase (GST), glutathione agarose beads (sulfur linkage), and glutathione were purchased from Sigma chemical company, St. Louis, MO. Stock solutions of PAF were dissolved in ethanol. PAF dilutions were subsequently made in culture medium. Kainate was prepared in Locke's solution as a 50 mM stock solution. Anti-JNK1 and p38 raised against polyclonal rabbit IgG were provided by Roger Davis, Howard Hughes Medical Institute, Worcester, Mass. In addition, anti-ERK1, anti-JNK1, and anti-p38 rabbit polyclonal IgG antibodies were purchased from Santa Cruz Biotechnology, Inc., Santa Cruz, CA.

Preparation of primary rat hippocampal cultures- Primary rat hippocampal cultures were prepared from modifications of Clark *et al.* (1992). Culture plates were coated with Matrigel (Collaborative Biomedical Products; Bedford, MA Research). Hippocampi were obtained from 1-3 day rat pups and pooled in oxygenated Liebovitz's (L-15) medium plus 0.05% bovine serum albumin (BSA). Hippocampi were then moved to a solution of 0.1% papain in L-15 + BSA for 20 minutes (under oxygen), then triturated with a Pasteur pipette. Cell yields are calculated using a hemocytometer and trypan blue. Cells were plated at 500,000 cells/ml in MEM plus 10% fetal calf and horse serum supplemented with insulin, transferrin and selenium, and glucose and glutamine, and maintained in 37° C, 5% CO_2 incubators. After 1 day in vitro (DIV), cultures were treated with 10^{-5} M AraC to control astrocyte proliferation. After 4 DIV, AraC was removed and cells moved to fresh MEM lacking serum. All experiments were carried out on cultures after 12 DIV.

Preparation of Astrocytes- Primary rat astrocyte cultures were prepared as described by DeCoster and Yourick, (1994). These cultures were grown in flasks and were not treated with AraC, thus allowing for astrocyte proliferation. Repeated exchanges of culture media containing fetal calf and horse serum was carried out on the cultures weekly. This reduces neuronal content (due in part to the glutamate content in serum) and further stimulated astrocyte proliferation. Once culture flasks reached confluence, cells were lifted from the flasks and dissociated with trypsin/EDTA treatment, centrifuged to a cell pellet, and then diluted in culture media and replated for experimental use. These cultures were then washed repeatedly with serum-containing media until reaching confluence before using for experiments.

PAF and Kainate Treatment of Primary Rat Hippocampus Neuronal Culture for Kinase Assay- Primary cultures of rat hippocampal neurons were grown on 6-well plates for at least 12 days as described in hippocampus culture section. Cells were treated by either a 1-hour exposure to new medium containing PAF or a fifteen-minute exposure to kainate in Locke's solution (plus 1 μM glycine, and without $MgCl_2$) at room temperature. In some conditions, cells were pre-treated with the antagonists BN50730 or CNQX before and during the incubation with agonists. At termination of treatment, cells were washed with cold PBS, scraped in homogenization buffer and homogenized. The homogenates were then centrifuged at 2800xg for 15 min at 4°C, and the supernatants were used for the assay.

In Vitro Immunocomplex Kinase Assay– After washing with cold PBS to terminate agonist stimulation, cells were scraped in PBS and homogenized, then centrifuged at 2800 X g for 15 min at 4 degrees C, and the supernatants used for the kinase assay. Plasmids containing GST-

cJun and GST-ATF2 were prepared as previously described (Smith and Johnson, 1988). Triton-X soluble protein lysates were pre-cleaned with Protein A agarose (Boehringer/Manheim) and then incubated with 1:100 dilutions of anti-JNK1 antibody, anti-p38 antibody, and anti-ERK1 or ERK2 antibody for 1-2 hours at 4°C. The immunoprecipitates were washed twice with homogenization buffer and once with kinase assay buffer (25 mM HEPES pH 7.4, 20 mM $MgCl_2$, 20 mM glycerophosphate, 10 mM sodium orthovanadate, and 2 mM dithiothreitol). Aliquots of 5 μg immunoprecipitates were incubated for 30 minutes at 30°C, using either 10 μg of GST-cJun fusion protein, 10 μg of ATF-2 cJun fusion protein, or 10 μg myelin basic protein (MBP, Uptstate Biomedical, New York), and 20 μM ATP (3 μCi, [^{32}P]ATP at 10 Ci/mM, Amersham Corp.; Arlington Heights, IL) in 40 μl kinase buffer as previously described (Hibi et al., 1993). The reactions were terminated by spotting 20 μl of sample onto P81 filter paper squares (Whatman; Maidstone, England) and simultaneously adding 10 μl of Laemmli buffer to the remaining samples and placing on ice. The filter papers were washed twice with 10 ml per square of 1% phosphoric acid followed by one wash with water. After counting, phosphorylation of substrate proteins by SDS-PAGE analysis was quantified using a phosphor imager (Bio-Rad; Hercules, CA).

Cytotoxicity Assay - Assays for cytotoxicity measured lactate dehydrogenase (LDH) release and were carried out as previously described (DeCoster et al., 1992). Cytotoxic treatments of cells treated with kainate were carried out at room temperature in Locke's solution without $MgCl_2$ and supplemented with 1 uM glycine. Cells were treated with kainate (diluted in Locke's solution) or control (Locke's solution alone) for 15 minutes. After cytotoxicity

treatment, Locke's solution was removed from cells and replaced with warm (37°C) minimal essential media (MEM) containing Earle's salts and no phenol red. Cultures were returned to the CO_2 incubator (37°C) overnight. 18-20 hours later, 50 μL of MEM-cell supernatant was assayed for LDH activity using Sigma Kit (228-UV) with the aid of a Beckman DU-640 spectrophotometer connected to a microcomputer. Percent neuroprotection by antagonists was calculated as previously described (DeCoster et al., 1995).

RESULTS:

Kainate activates ERK, JNK, and p38 kinases in hippocampal neurons and is blocked by the PAF receptor antagonist BN 50730. Kainate induced epileptogenesis prominently enhances the expression of genes *in vivo* and a PAF receptor antagonist inhibits this effect (Marcheselli and Bazan 1996). Also, PAF potently activates stress sensitive MAP kinases in hippocampal neurons (Mukherjee et al., 1995). Therefore, we tested whether kainate could activate these MAP kinases and assessed the effect of the PAF antagonist BN 50730 on this activation, with the purpose of defining if PAF could be a messenger. Kainate at 5 μM activated ERK, JNK, and p38 kinases, with maximal stimulation at 100 μM kainate of 3-4 fold over controls for JNK and p38 kinases, and approximately 20-fold over controls for ERK (Figure 1). Both the PAF receptor antagonist BN 50730 and the AMPA/Kainate receptor antagonist CNQX almost completely blocked the activation of ERK, JNK and p38 kinases by kainate.

Co-administration of kainate and PAF to hippocampal neurons does not result in additive activation of ERK, JNK, or p38 kinase. The ability of the PAF receptor antagonist BN 50730 to block the activation of protein kinases elicited by PAF (Mukherjee et al., 1995) or kainate (Figure 1), indicated that PAF and kainate might utilize similar or shared pathways for this activation. Furthermore, since the AMPA/kainate receptor antagonist CNQX did not interfere with PAF activation of the kinases (Mukherjee et al., 1995), PAF might appear downstream of kainate in putative shared pathways. To further test this hypothesis, we added submaximal or near-maximal concentrations of PAF and kainate together to hippocampal neurons to investigate whether activation of ERK, JNK, and p38 kinases would be additive. As shown in Figure 2, co-administration of PAF and kainate was not additive for submaximal or near-maximal agonist concentrations for any of the kinases.

MAP kinases are activated by PAF and kainate in hippocampal neurons but not in astrocytes. Since our neuronal cultures contain a small percentage of astrocytes, we determined whether PAF and kainate stimulation of MAP kinases which we measured were due to activation in glia. In pure astrocyte cultures, neither PAF nor kainate activated the MAP kinases, while cultures of hippocampal neurons treated during the same experiment showed activation (Figure 3).

Antagonism of PAF and kainate receptors inhibits kainate-induced neurotoxicity in hippocampal neurons. To investigate the long-term consequences of kainate activation of MAP kinases in hippocampal neurons, cells were treated with 50 μM kainate using identical conditions as for MAP kinase experiments. For toxicity determinations, after 15 minutes of kainate stimulation, cells were returned to incubators overnight as described in Methods, and lactate

dehydrogenase (LDH) release assayed the following day. As shown in Figure 4, both the PAF receptor antagonist BN 50730 and the kainate receptor antagonist CNQX demonstrated neuroprotection against kainate toxicity, although BN 50730 was only slightly protective.

DISCUSSION:

In previous studies PAF has been shown to activate MAP kinases using cells lines, transfected cells, or non-neuronal cell types (Bonaccorsi et al., 1997; Honda et al., 1994; Fouda et al., 1995). We have recently shown that PAF potently and directly activates ERK, JNK, and p38 kinase in rat primary cultured hippocampal neurons (Mukherjee et al., 1995). This activation by PAF is almost completely blocked by the PAF receptor antagonist BN 50730. Since activation of the kinases by PAF is not blocked by the kainate receptor antagonist CNQX (Mukherjee et al., 1995), the bioactive lipid action is downstream of kainate receptors in hippocampal neurons.

Although Kurino et al. (1995) demonstrated MAP kinase activation by glutamate in rat hippocampal neurons, they found that non-NMDA receptors were not involved in this activation. In contrast, we found that kainate activated ERK, JNK, and p38 kinase in hippocampal neurons. It was of interest to note that kainate activation of these kinases was blocked by the PAF receptor antagonist BN 50730. While CNQX blocked kainate-stimulated activation of the MAP kinases (Figure 1), CNQX did not interfere with PAF stimulation of the kinases (Mukherjee et al., 1998). These results suggest that kainate and PAF may utilize a common pathway for activating the MAP kinases in hippocampal neurons, with kainate upstream of PAF in this pathway. This hypothesis is further supported by the experiments showing that co-administration of PAF and kainate does not result in additive activation of the kinases (Figure 2).

Previous work *in vivo* in our laboratory has shown that kainate induction of the early response gene for cyclooxygenase-2 is blocked by a PAF receptor antagonist (Marcheselli and Bazan, 1996), consistent with the idea that kainate is upstream of PAF receptor activation, and that kainate may stimulate PAF synthesis.

PAF has been shown to increase glutamate release and electrophysiological activity at the synapse (Clark et al., 1992; Gilboe et al., 1991; Kato et al., 1994, Wierasjko, 1993). Treatment of neuronal cell lines and primary neuronal cultures with PAF has been implicated to be neurotoxic, (Kornecki and Ehrlich, 1988; Nogami et al., 1997), however, long-term treatment and high concentrations of PAF were necessary for expression of this toxicity. In other studies, PAF antagonists have demonstrated some neuroprotection against excitotoxic and ischemic injury (Gilboe et al., 1991; Panetta et al., 1987; Prehn and Krieglstein, 1993). Our results in the present work indicate no neurotoxic action of PAF itself under conditions sufficient to elicit activation of the MAP kinases (data not shown), while kainate was toxic, causing release of LDH by the subsequent day. The toxicity of kainate was effectively blocked by the kainate-receptor antagonist CNQX, and to a lesser extent, by the PAF receptor antagonist BN 50730 (Figure 4). Since neither PAF nor kainate stimulated activation of the MAP kinases in pure astrocyte cultures (Figure 3), the results reported here appear to be of neuronal origin.

Synaptic efficacy in the hippocampus in the form of LTP have recently been shown to be accompanied by activation of p42 MAP kinase (English and Sweatt, 1996). Therefore, receptor mediated activation of stress sensitive MAP kinases through PAF may be a physiological modulatory event that, when overactivated, leads to neuronal damage.

ACKNOWLEDGEMENTS

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FIGURE LEGENDS:

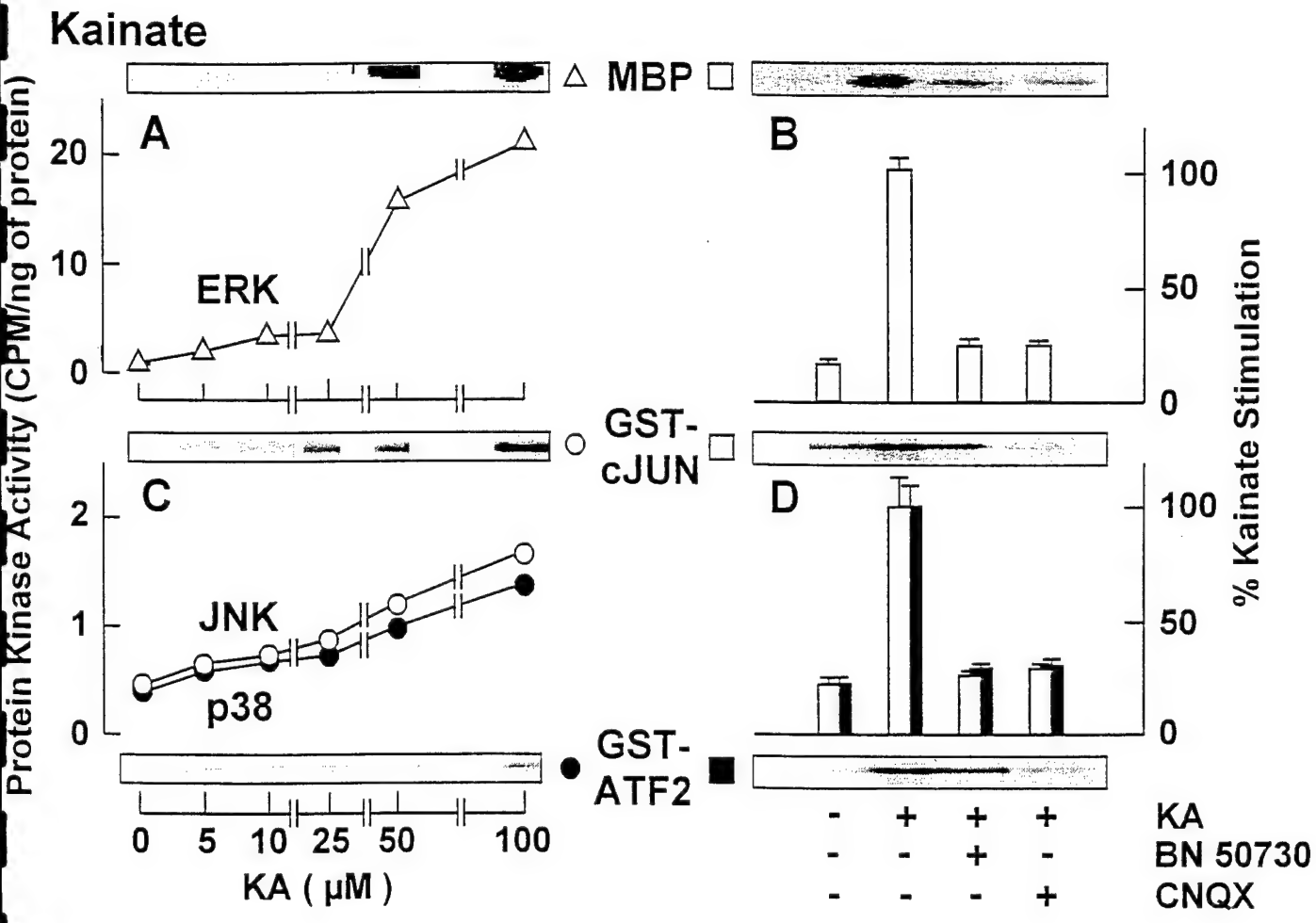
Figure 1. Kainate activates ERK, JNK, and p38- MAP kinases in primary hippocampal neurons. Cells were treated with kainate at the indicated concentration (A and C) or at 50 μM (B and D) and activation of protein kinases determined using different substrates as described in Methods. Autoradiogram on each figure is a representative gel. Line graphs (A and C) show the quantification of the autoradiograms. Panels A and C: concentration response curves for kainate activation of ERK (panel A) and JNK and p38 kinases (panel C). Panels B and D: antagonism of kainate activation of MAP kinases. Data have been normalized to activation elicited by 50 μM kainate, which has been set at 100%. As indicated, some cells were pretreated with BN 50730 (1 μM) or CNQX (5 μM) as described in Methods. Data shown are representative experiments from multiple platings of cells. Averages are plotted with standard errors shown in panels B and D.

Figure 2. Combinatorial effects of kainate and PAF activation of MAP kinases in primary hippocampal neurons is less than additive. Cells were treated with PAF and/or kainate at indicated concentrations and MAP kinase activity measured as described in methods. Shaded bars indicate ERK-, white bars JNK-, and black bars p38-kinases, respectively. Data are averages from a representative experiment with standard errors shown. Where error bars do not appear, error was less than the width of the top bar line.

Figure 3. Comparison of kainate and PAF activation of MAP kinases in primary hippocampal neurons and astrocytes. PAF (100 nM) or kainate (50 μM) was added to primary cultures of

hippocampal neurons or astrocytes and MAP kinase activity measured as described in Methods. Hatched bars indicate ERK-, white bars JNK-, and black bars p38-kinases, respectively. Data shown are averages from representative experiments of multiple platings of cells.

Figure 4. CNQX and BN 50730 inhibit kainate-induced neurotoxicity in hippocampal neurons. Hippocampal neurons were treated with 50 μM kainate in the presence or absence of the indicated antagonists and lactate dehydrogenase release assayed and percent neuroprotection calculated as described in Methods. BN 50730 concentration was 1 μM and CNQX was 5 μM. Data shown are averages from multiple platings of cells with standard errors shown. *= $p<0.05$ by Student's t-test.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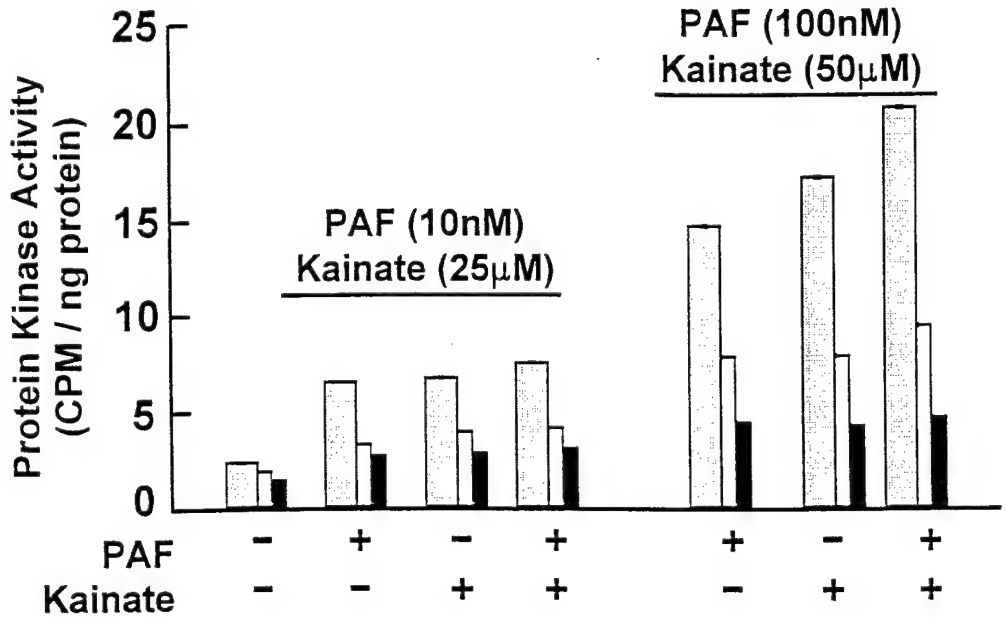
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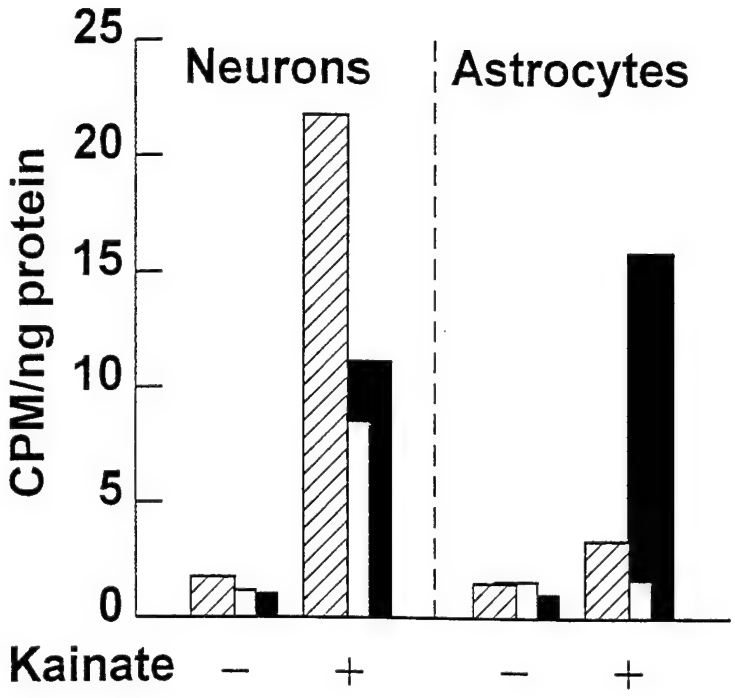
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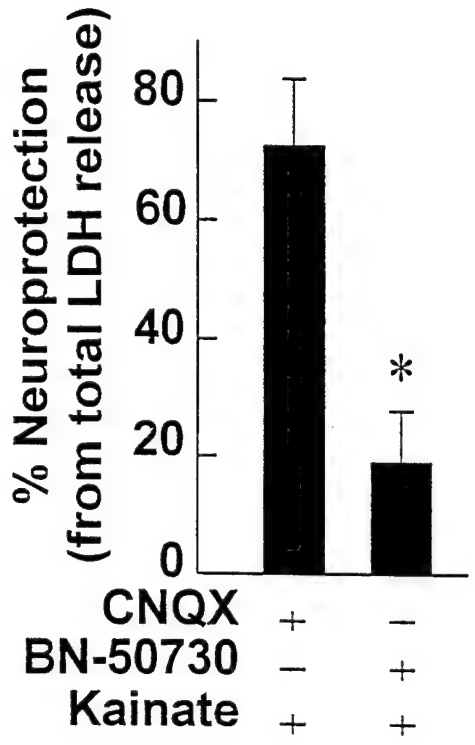
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Kainate + +

Recombinant plasma-type platelet-activating factor acetylhydrolase attenuates NMDA-induced hippocampal neuronal apoptosis.

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ABSTRACT

The bioactive lipid platelet-activating factor (PAF) accumulates in brain during injury, seizures and ischemia and may, in addition, be significant in AIDS dementia and in other neurodegenerative diseases. We have used plasma-type recombinant PAF acetylhydrolase (rPAF-AH) to test the hypothesis that PAF accumulation is involved in early events leading to neuronal apoptosis during excitotoxic neuronal injury. Neuronal cultures were labeled with FITC-12-dUTP (TUNEL technique) and propidium iodide, digitized using fluorescence microscopy and a chilled 3CCD color camera, and analyzed with 2D graphics analysis software. N-methyl-D-aspartate (NMDA) (50 μM, 2 h) induced a 2.5-fold increase in apoptosis of hippocampal neurons compared with controls when analyzed 24 hours after NMDA treatment. Hippocampal neurons receiving rPAF-AH (20 μg/ml) before, during, and after NMDA treatment demonstrated a concentration-dependent neuroprotective effect which resulted in 47% and 30% neuroprotection against 50 and 100 μM NMDA, respectively. The non-competitive NMDA receptor antagonist MK-801(300 nM) completely inhibited apoptosis caused by NMDA. The neuroprotective effect of rPAF-AH against NMDA-induced apoptosis was confirmed using as additional criteria, histone release, electron microscopy and DNA laddering. Neuroprotection elicited by rPAF-AH demonstrates that PAF is an injury mediator in NMDA-induced neuronal apoptosis and that the recombinant protein is potentially useful as a therapeutic approach.

Key words: apoptosis, neuroprotection, platelet-activating factor acetylhydrolase, NMDA receptor

INTRODUCTION

Platelet activating factor (PAF, 1-0-alkyl-2-acetyl-sn-glycerophosphocholine) is a membrane-derived bioactive phospholipid generated via phospholipase A_2 (PLA_2) activation, as well as other pathways (Prescott et al., 1990; Bazan, 1995). This bioactive lipid may be significant in the pathophysiology of brain trauma, seizures and ischemia (Doucet et al., 1990) and may, in addition, be a key participant in AIDS dementia (Gelbard et al., 1994; Epstein et al., 1993) and in other neurodegenerative diseases (Lipton, 1994). PAF is physiologically involved in excitatory neurotransmitter release, neuronal plasticity, and memory formation. PAF enhances excitatory neurotransmitter release through a presynaptic receptor (Clark et al., 1992), is a retrograde messenger in LTP (Wieraszko et al., 1993), and upregulates memory formation, both in the hippocampus (Izquierdo et al., 1995) and in the striatum (Packard et al., 1996). This bioactive lipid also induces fos/JUN/AP1 signaling (Squinto et al., 1989) and cyclooxygenase-2 (COX-2), the inducible prostaglandin synthase (Bazan et al, 1994; Marcheselli et al., 1996). Therefore, although low concentrations of PAF modulate cell function, relatively higher concentrations may become neurotoxic. These dual properties are also shared by other neuroactive molecules, including the neurotransmitter, glutamate.

PAF is inactivated by cleavage of the *sn*-2 acetyl residue by the serine esterases, PAF-acetylhydrolases (PAF-AH). The are two main isoenzymes: a) plasma type, a dimeric complex associated primarily with low-density lipoproteins (Stafforini et al, 1991; 1993; Tjoelker et al., 1995; Stafforini et al, 1997; Cao et al., 1998); and, b) cytosolic isoenzymes (Hattori et al., 1993; 1994a,

b; Matsuzawa et al., 1997), that include brain-specific intracellular PAF-AH consisting of two subunits with catalytic activity and a presumably regulatory subunit (Hattori et al., 1993; 1994b). The gene encoding the latter corresponds to the LIS-1 gene, mutated in Miller-Dieker lissencephaly (Hattori et al., 1994a).

The human plasma form of PAF acetylhydrolase has recently been cloned and the recombinant protein (rPAF-AH) is effective in blocking inflammation in non-neural cells (Tjoelker et al., 1995). Here we investigated if NMDA-induced apoptosis in hippocampal cell culture is inhibited by rPAF-AH. A concentration-dependent neuroprotection by rPAF-AH on hippocampal cells was found using several criteria to evaluate apoptosis: the TUNEL staining technique, histone release, DNA laddering and electron microscopy. Part of these results have appeared in abstract form (Ogden et al., 1997a, b).

MATERIALS AND METHODS

Primary hippocampal neuron preparations:

Hippocampal cultures were prepared from methods described by Clarke et al. with modifications. In brief, hippocampi from 1 - 3 day old rat pups were dissected under aseptic conditions and moved to sterile oxygenated albumin dissolved in Leibovitz's L-15 medium. Culture media consisted of minimal essential media (MEM, glutamine-free) supplemented with 10% fetal calf and 10% horse sera, 10 μg/mL insulin, 200 uM glutamine, 6 mM glucose, and 50 units/mL penicillin/streptomycin. Cells were plated on Matrigel-coated culture plates. Cells were maintained in 5% CO_2 at 37 degrees C. At one day *in vitro* (1 DIV), 1 x 10^{-5} M cytosine arabinoside was added

to the cultures to inhibit glial proliferation. Cell media was removed at 4 DIV and replaced with serum-free culture media. All experiments were conducted on cells 14-28 DIV.

Primary astrocyte preparations:

Pure astrocyte cultures were prepared from cells isolated as described above, but plated on flasks without cytosine arabinoside treatment, and were washed with media plus serum three times at 4, 9, and 15 DIV. This process depletes the cultures of neurons resulting in cultures that are approximately 95% astrocytes, as described previously (DeCoster et al., 1994).

NMDA treatment:

NMDA (diluted in MEM) at the indicated concentration was added directly to cell cultures by adding a 20x sample in the appropriate volume. Controls received the same volume of MEM only. NMDA remained on cells for 2 hours at 37° in a 5% CO_2 incubator. NMDA and media in all other wells was then removed and replaced with MEM and cells returned to 37° in a 5% CO_2 incubator until the following day when apoptosis analysis was performed. rPAF-AH treatment included a 30-minute pretreatment time at 37° C with 20 μg/ml of enzyme rPAF-AH was allowed to stay on the cells during the NMDA treatment phase, and it was replaced in MEM following agonist treatment at a concentration of 10 μg/ml of enzyme. MK-801 treatment (300 nM) was performed with a 10-minute pretreatment period at 37° C. The compound remained in media through the NMDA

treatment phase and was replaced in MEM following treatment with the agonist. NMDA was obtained from Sigma Inc. (St. Louis, MO). rPAF-AH was provided by ICOS Corp. (Bothell, WA). MK-801 was obtained from RBI Inc. (Natick, MA).

FITC-dUTP TUNEL staining and analysis of images:

The staining of cells for apoptosis was performed using the Apoptosis Detection System, Fluorescein by Promega (Madison, WI). The cells were counter-stained with propidium iodide (Sigma; St. Louis, MO). The stained cells were then digitized using fluorescence microscopy and a Hamamatsu chilled 3-CCD color camera. Following capture of the images, the cells were counted using Image Pro Plus (Media Cybernetics, Silver Spring, MD) two-dimensional graphics analysis software. Fluorescein-positive cells were counted by the software. The data were exported to Microsoft Excel, then expressed as the ratio of the number of apoptotic (green) cells compared to control (red) cells. Percent protection by rPAF-AH or MK-801 against NMDA was calculated in Excel using the following formula:

$$\% \text{ Protection} = (((NMDA_r - Control_r) - (Experimental_r - control_r))/(NMDA_r - Control_r))*100$$

Where:

$NMDA_r$= the ratio of green cells: red cells for a field treated with NMDA

$Control_r$= the ratio of green cells: red cells for a control field

$Experimental_r$= the ratio of green cells: red cells for a field treated with NMDA and

rPAF-AH or MK-801

/= division

*= multiplication

Histone release ELISA:

Cell culture samples were homogenized in a phosphate buffer containing protease inhibitor cocktail: 0.5 mM PMSF, 2 μg/ml Aprotinin, 2 μg/ml leupeptin, 2 μg/ml trypsin inhibitor (soy bean). Samples were diluted 10 fold with a lysis buffer provided by the ELISA kit (Boehringer Mannheim, Germany, Cat.# 1774425). Sample aliquots in 20 μl volume were applied on 96 well plates, and incubated for two hours with 80 μl of incubation buffer, containing the monoclonal antibodies directed against DNA and histones for detection of mono and oligo-nucleosomes. After washing off unbound antibodies, the immunocomplex of DNA-histone-antibodies, remain bound to the streptavidin coated plates, where detection is obtained through horseradish-peroxidase reaction. Quantitative analysis was performed in a Spectramax-250 plate reader (Molecular Devices; Sunnyvale, CA).

Electron Microscopy:

After treatment with NMDA and/or antagonists as indicated above, neuronal cultures were prepared for electron microscopy by removing media from cells, scraping them from the surface of the culture dish, and pelleting by brief centrifugation. The cell pellet was then fixed with 2% glutaraldehyde, 2% formaldehyde, 0.135 M sodium cacodylate buffer (pH 7.3) overnight at 4° C.

The pellet was then rinsed three times in cacodylate buffer and post-fixed for 15 min in 1% OsO_4 in cacodylate buffer. After rinsing three more times in cacodylate buffer, the pellet was run through an ethanol dehydration series (50-100%). Infiltration and plastic embedding occurred in a mixture of Epon-araldite plastic. Silver-gold sections were placed on nickel grids and contrasted with uranyl acetate and lead citrate. Sections were viewed with a Zeiss C10 transmission electron microscope, and photographed on 3.25" x 4" Kodak electron microscope film 4489 (EMS, Ft. Washington, PA).

DNA Laddering:

Neuronal cellular DNA was extracted using the method of Tso et al. and electrophoresed on 1.8% agarose-TBE gels. A 100 base pair DNA ladder (Gibco BRL, Gaithersburg, MD) was used as a positive control for apoptosis. Images of ethidium bromide-stained gels were captured using a Gel Doc 1000 video system with Molecular Analyst software (BioRad; Hercules, CA).

RESULTS

Neuronal apoptosis in primary rat hippocampal cultures induced by NMDA was assessed by TUNEL-staining image analysis (Fig 1). rPAF-AH added prior to, during, and following NMDA treatment reduced apoptosis. rPAF-AH resulted in significant protection against both 50 and 100 μM NMDA-induced apoptosis comparing the ratio of TUNEL positive vs negative cells (Fig 1). PAF-AH decreases 50 μM NMDA-induced apoptosis and, to a lesser extent, apoptosis induced by 100 μM NMDA (Fig 1). It appears from these data that 50 μM NMDA under these conditions elicits a nearly maximal level of apoptosis, as 100 μM NMDA yields similar results. No injury was

detected by NMDA addition to pure astrocytes, indicating that the effect of rPAF-AH on apoptosis in the current study was neuronal. Application of rPAF-AH alone did not affect neuronal survival (data not shown). Neuronal cultures using the TUNEL staining technique with propidium iodide counterstain were subjected to a line analysis on the indicated fluorescence range. In figure 2, the fluorescence histogram above each field of cells depicts the relative signal strength of damaged vs non-damaged DNA as indicated by the green and red lines, respectively. Peaks represent cell profiles. This analysis demonstrates the ratio of damaged versus non-damaged DNA in each cell area. Note that the NMDA treated condition shows consistently the highest ratio of green:red signal; rPAFAH lowers this ratio, while cells treated with the non-competitive NMDA antagonist MK-801 and control cells have the lowest ratio. Using identical exposure conditions and staining techniques, it was found that astrocyte cultures exposed to 50 μM NMDA showed no TUNEL-positive staining, while all cells were stained with propidium iodide (data not shown), indicating that apoptotic damage measured in the present work was neuronal.

Table 1 summarizes the inhibition of NMDA-induced apoptosis by rPAF-AH or MK-801 using the TUNEL staining technique, to derive the number of TUNEL positive and negative cells from several experiments. rPAF-AH significantly inhibited 50 μM- and 100 μM-NMDA induced apoptosis by an average of 47 and 30%, respectively, when all experiments were considered. As a positive control, the non-competitive NMDA receptor antagonist MK-801 completely blocked NMDA induced apoptosis at both 50 and 100 μM NMDA.

To further ascertain apoptosis, after exposure to NMDA with either rPAF-AH or MK-801, neuronal cultures were analyzed by electron microscopy. Control cells displayed intact nuclei with

homogeneous content (Figure 3A). In contrast, cells treated with 50 μM NMDA showed marked compromise of nuclear membranes including blebbing, condensation of chromatin, and overall shrinkage of the nuclei (Figure 3B). The presence of rPAF-AH during NMDA treatment provided protection from the apoptotic events as evaluated by electron microscopy. Of seven nuclei observed, only one showed damage (data not shown). Additionally, two other methods were used to determine the induction of apoptotsis by NMDA and protection of neurons by rPAF-AH. As shown in Figure 4A, compared to control cells, cultures treated with 50 μM NMDA, demonstrated more fragmented DNA by DNA laddering, with rPAF-AH treatment inhibiting this process. Finally, using a histone antibody ELISA, nucleosomal fragmentation and release of histone-associated DNA was increased by 50 μM NMDA treatment of neuronal cultures, and rPAF-AH markedly decreased this process (Figure 4B).

DISCUSSION:

The accumulation of PAF in the nervous system promotes neurotoxicity (Bazan et al., 1995; Prehn et al., 1993; Kornecki et al., 1988). PAF receptor antagonists are neuroprotective in certain experimental models, such as ischemia-reperfusion (Prehn et al., 1993, Panetta et al., 1987; Gilboe et al., 1991). However, these observations have not been translated to clinical studies in humans, due to a variety of factors. One possible factor compounding PAF receptor antagonist action is the role of oxidized phospholipids. These phospholipids contain fragmented polyunsaturated fatty acyl residues in the *sn*-2 position which allows binding to the PAF receptor. In many instances, oxidized

phospholipids are generated in larger quantities than PAF. These oxidized phospholipids could potentially quantitatively overwhelm receptor antagonists (Bazan, 1995).

Here we show an alternative to blocking the PAF receptor for neuroprotection: plasma-type PAF-AH, an enzyme that breaks down PAF. This recombinant enzyme can effectively inhibit NMDA-induced apoptosis in primary hippocampal neurons. Very recently, it was shown that another type of PAF-AH, intracellular PAF-AH II exerts cytoprotection against oxidative stress-induced apoptosis in a non-neuronal cell line (Matuszawa, 1997). Through the use of fluorescence staining with the TUNEL technique and quantitative image analysis, we have demonstrated that rPAF-AH significantly attenuates NMDA-induced apoptosis. The neuroprotection was demonstrated both by quantifying the number of TUNEL positive cells (apoptotic), as well as by line analysis profiles of cell areas, which indicate the ratio of damaged to non-damaged nuclear material (Figure 2). As shown in Figure 2, this type of analysis can be applied to generate profiles of individual, or groups of cells. While this approach supports the evidence that rPAF-AH inhibited NMDA-induced apoptosis in neurons (as did MK-801), line analysis may be more effectively used in the future to trace the time course of apoptosis generation. In the current work, image analysis was carried out on one time point (2 hours of NMDA treatment at 37°C with 24 hours of recovery time) under different treatment conditions. The number of damaged cells (nuclei) were then quantified. As demonstrated in Table 1, this technique allowed large numbers of cells to be analyzed.

Additional assessments by different techniques including electron microscopy, confirmed and extended our findings. Nuclear shrinkage, chromatin condensation, and DNA blebbing triggered

by NMDA treatment were absent in control nuclei and in cells treated with rPAF-AH plus NMDA. DNA laddering and histone release further demonstrated the protective effect of rPAF-AH against NMDA-induced neuronal apoptosis. Although these latter two procedures appeared less distinct than the TUNEL technique and electron microscopy, this may be due to the more extreme stages of apoptosis required for a clearer laddering effect or release of histones. While more extensive degradation of neuronal genomic DNA might be required for enhancement of DNA laddering and histone release, under our experimental treatment conditions, this extent of damage did not appear to occur. In this regard, the TUNEL technique was a sensitive indicator. The technical challenge of sample preparation and obvious high magnification used in electron microscopy limit the large numbers of nuclei that can be evaluated by this method compared to image analysis using conventional microscopy. Therefore, multiple methods of assessing apoptosis and neuroprotection are very valuable.

The use of rPAF-AH represents an alternative to PAF antagonists to control pathological PAF accumulation that takes place in excitotoxicity, such as in stroke, epileptic brain damage, neurotrauma and neurodegenerative diseases. Recombinant PAF-AH delivery should follow physiological transport mechanisms, and should show pharmacokinetics similar to the natural protein. The plasma PAF-AH is also characterized by a resistance to proteolytic attack. The ability for PAF-AH to hydrolyze oxidized phospholipids that possess PAF-like activity may be an important aspect of neuroprotection by this enzyme. Brain phospholipids are enriched in polyunsaturated fatty acids. Free radical damage and fragmentation of the C_2 polyunsaturated fatty acid residue of phosphatidylcholines yields species that have a short residual fatty acyl chain in the C_2 position.

These molecules have structural resemblance to PAF and, therefore, have biological activity at the PAF receptor (Smiley et al., 1991; Zimmerman et al., 1994; Heery et al., 1995). Although these PAF-like species are not well characterized in brain, they may have a significant role in neuronal damage. Further investigation into the physiological significance of PAF and similar phospholipids in the nervous system is certainly warranted. Currently our laboratory is exploring the potential signal transduction mechanisms of this neuronal injury cascade as an indication of key target steps for therapeutic intervention.

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FIGURE LEGENDS

Figure 1: rPAF-AH protects against NMDA-induced apoptosis in hippocampal neurons as assessed by TUNEL technique and cell counts. Neurons were treated with rPAF-AH and/or NMDA as

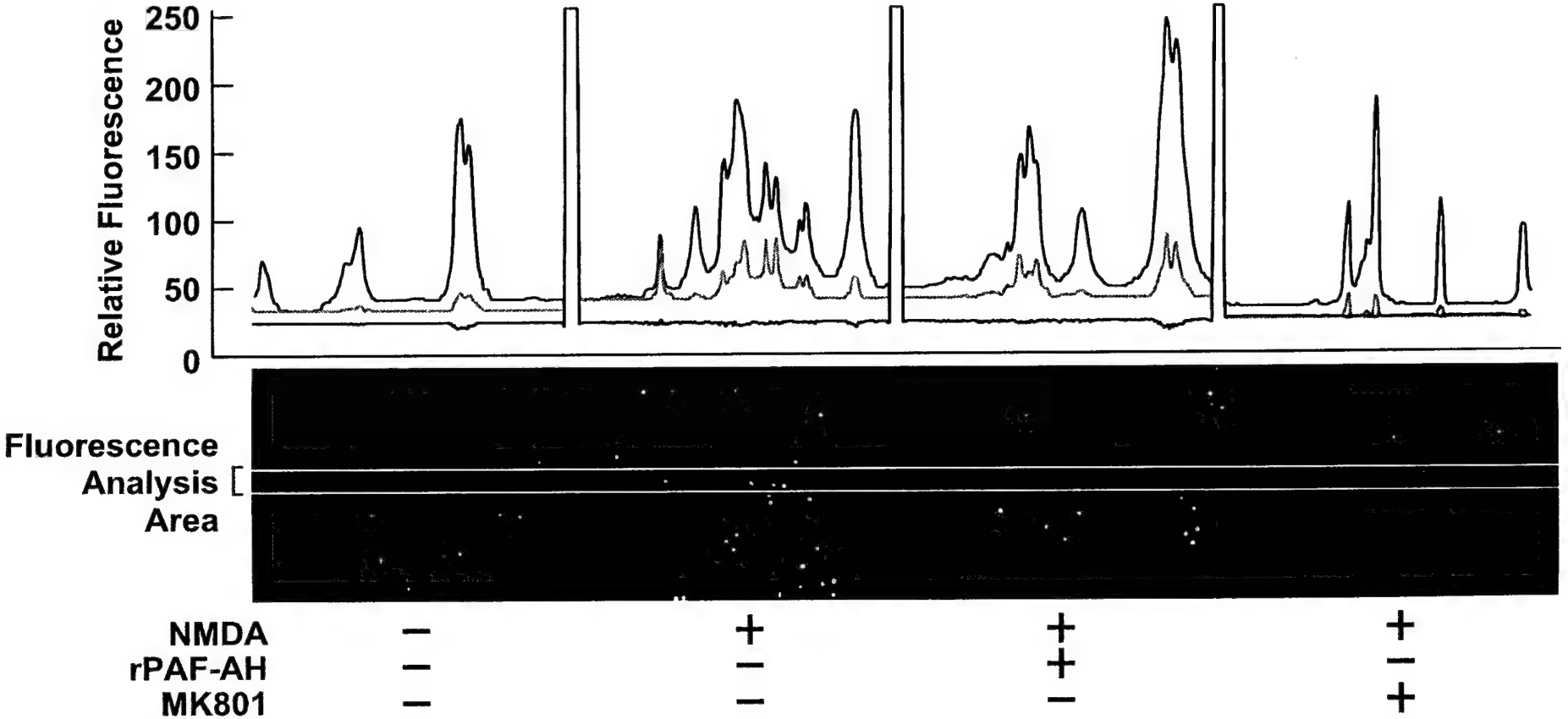
described in methods. Apoptosis was quantified using image analysis of cells stained green by fluorescein via the TUNEL technique (damaged) and counter stained red with propidium iodide (undamaged). The ratio of cells in each category (green:red) is shown times a factor of 100. Data shown are from two representative experiments, with each condition the average ratio from multiple fields and multiple culture wells. Standard deviation bars are shown. **=P< 0.005; *= P<0.02 by Student's t-test.

Figure 2: rPAF-AH or MK-801 protect against 50 μM NMDA-induced apoptosis in hippocampal neurons as assessed by TUNEL technique and damaged/undamaged DNA line profiles. Cells were treated with rPAF-AH, NMDA, and/or MK-801 as described in methods. Cells were stained as described in Figure 1. Digitized images show representative fields of stained cells in each condition (original microscope magnification of all fields= 200x). Using Image Pro Plus software, a line analysis was carried out on the region indicated as fluorescence analysis area. The resulting histogram above each image indicates the profile of green (damaged) and red (undamaged) nuclear material for each cell or group of cells. Peaks indicate individual or groups of cells. Note that on average, the ratio of damaged/undamaged DNA is highest in NMDA treated cells (second panel). rPAF-AH decreases this ratio (third panel), with controls and MK-801 showing the lowest ratio of green:red.

Figure 3: rPAF-AH protects against 50 μM NMDA-induced apoptosis in hippocampal neurons: electron microscopy evaluation. Cells were treated with rPAF-AH and/or NMDA and processed for

electron microscopy as described in methods. Figure 3A shows a control nucleus. Figure 3B shows nucleus from cultures treated with NMDA. Note the shrunken appearance of the nucleus, with prominent condensation of chromatin and DNA blebbing. Original microscopic magnification of all figures= 4,000x.

Figure 4: rPAF-AH protects against 50 μM NMDA-induced apoptosis in hippocampal neurons: DNA laddering and histone release ELISA. Cells were treated with rPAF-AH and/or NMDA as described in methods. In panel A, control cells (C) and cells treated with NMDA (N) or NMDA plus rPAF-AH (N+P) were processed for DNA laddering as described in methods. The density of each band has been converted to a spectral scale, with black and blue indicating low, and green, yellow, and red indicating high, density. Note that for equal amounts of DNA loaded on the gel, the DNA laddering pattern is less intense for the N+P lane compared to the N lane. The left lane is a 100 base pair ladder standard. In B, a representative experiment using an ELISA of histone-DNA release from hippocampal cell cultures treated with NMDA and/or rPAFAH. *$P<0.03$ by Student's t-test.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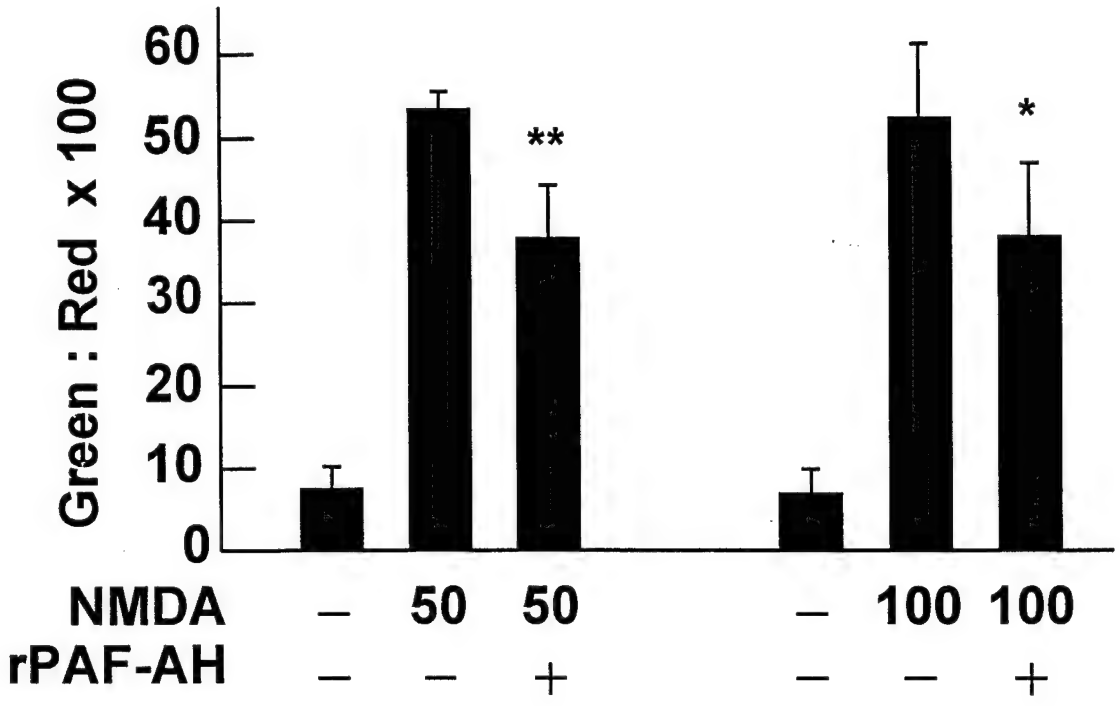
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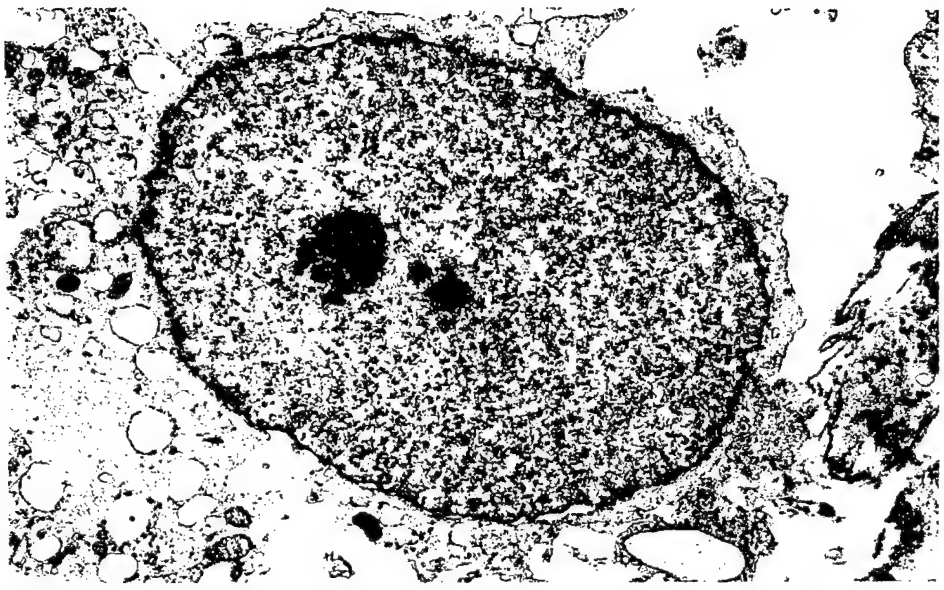

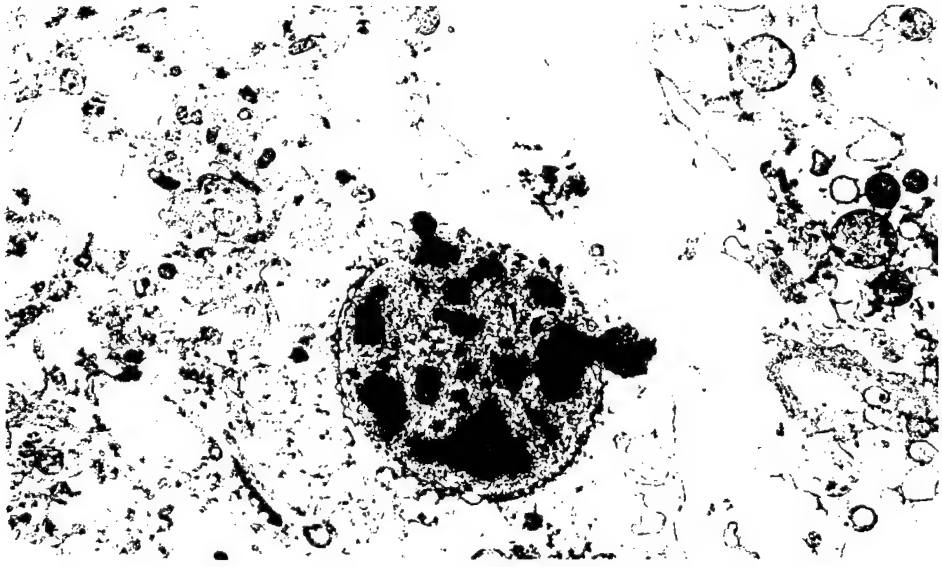

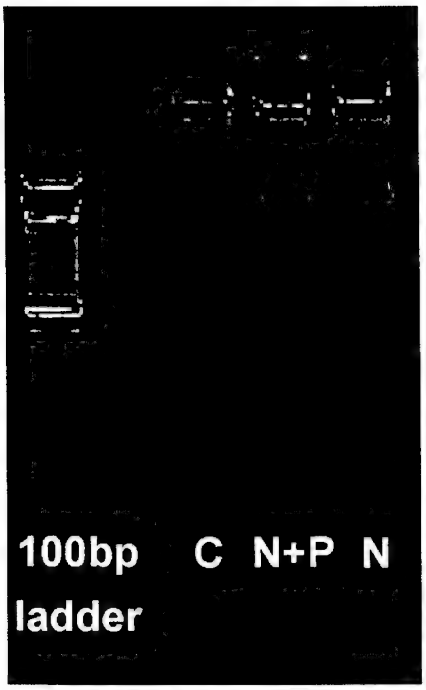
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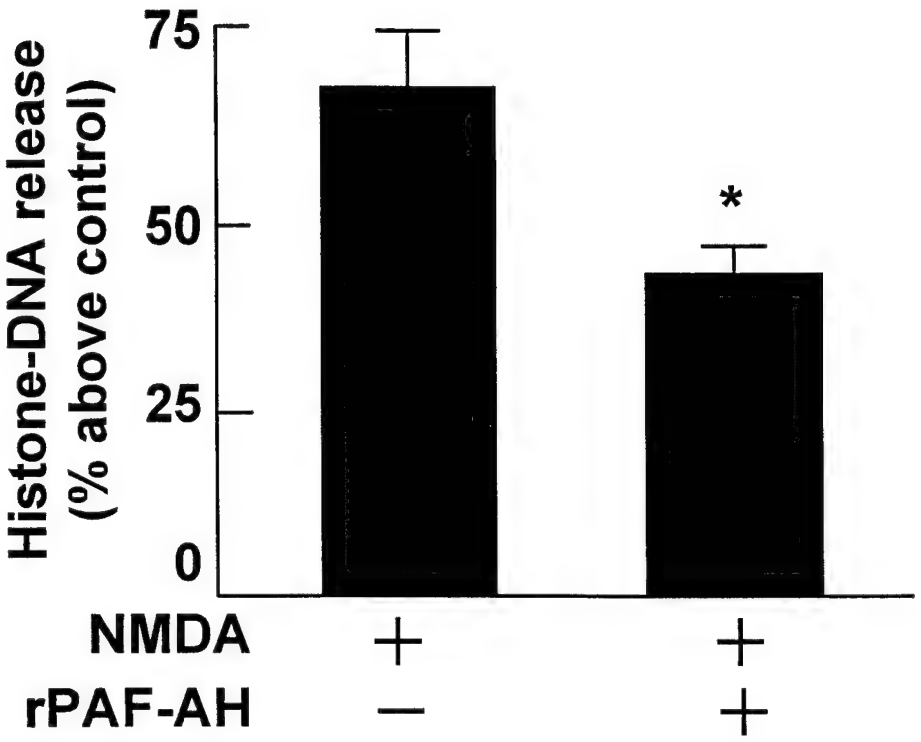
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Bioactive Lipids and Gene Expression in Neuronal Plasticity

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INTRODUCTION

Excitable membranes are a reservoir of bioactive lipids that are contained as part of the structure of phospholipids. Signals such as those elicited by receptor occupancy trigger, via the activation of phospholipases, the release of phospholipid moieties. These enzymes are under control of receptors, calcium ions, protein kinase/phosphatase cascades, and of other signaling mechanisms which still need to be better characterized. The best defined regulatory events of these enzymes are those controlled by the seven transmembrane domain superfamily of receptors that, through G proteins, activate phospholipase C (PLC). This enzyme predominantly hydrolyzes phosphatidylinositol 4', 5' bisphosphate (PIP_2), resulting in the production of the second messengers, diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). These two messengers of inositol lipids represent a bifurcating, informational system inside the cell. The synaptic inositol lipid signaling system is activated by neurotransmitters, growth factors and drugs. IP_3 promotes intracellular Ca^{2+} mobilization by binding to a specific receptor in the endoplasmic reticulum, which in turn enhances calcium-dependent cellular processes.

The other product of inositol lipid degradation, DAG, is an activator of protein kinase C (PKC). PKC plays a modulatory role by catalyzing the phosphorylation of specific proteins that contribute to cell function. The modulation of cell function by PKC is complex because a) there are several isoforms of this enzyme, some are brain specific (Braquet et al., 1987); b) also, phospholipase A_2 (PLA_2) products such as free arachidonic and docosahexaenoic acids modulate some protein kinases C and c) there is a high degree of synergy and feedback modulation between various intracellular pathways of cell signal transduction, including also other protein kinases. Synaptic plasticity involves changes in PKC activity, hence phospholipases may play a

role in regulating the formation of neuronal pathways.

In addition to the PLC-inositol signaling pathways, PLA_2 is an important modulator of the release from membrane phospholipids, of arachidonic acid and platelet-activating factor. PLA_2 cleaves the fatty acyl chain at the C2 position of a phospholipid. In excitable membranes, the C2 acyl chain of phospholipid is often polyunsaturated and, when cleaved, provides a substrate for oxidative metabolism (arachidonic acid) to biologically active lipid mediators. In addition, there is, in excitable membranes, a small, but metabolically active pool of 1-alkyl-2-acyl-glycero-3-phosphocholine. The 2-acyl is often arachidonic acid. The lyso-phospholipid PLA_2 product of this phospholipid is a precursor for the potent lipid mediator platelet-activating factor (PAF). Therefore, PLA_2 may release both arachidonic acid and the PAF precursor. Here the potential significance of PLA_2 and particularly of PAF in neurotransmission, gene expression, and neuronal plasticity are described.

Platelet-Activating Factor

Platelet-activating factor (PAF, 1-O-alkyl-2-acetyl-glycero-3-phosphocholine), an acetylated alkyl ether phospholipid, is one of the most potent of all known bioactive lipids. PAF was initially described, as its name implies, as a leukocyte-derived mediator that induced the aggregation and activation of platelets (Henson, 1970; Benveniste et al., 1972). Today, it is known that there are there are wide-ranging functions for PAF, both as a mediator of inflammatory and immune responses, and as a second messenger involved in physiological and pathophysiological events of intracellular communication(Benveniste et al., 1972; Prescott et al., 1990; Snyder, 1995).

PAF is rapidly produced in brain in response to ischemia and seizures (Squinto et al.,

1981; Panetta et al., 1987) and in neuronal cell cultures in response to neurotransmitters (Sogos et al., 1990). Neurons and glial cells have the capacity for stimulus-evoked PAF synthesis.

There are at least three different routes for PAF biosynthesis (**Figure 1**). These involve either the modification of the membrane phospholipid precursor alkylacyl glycerophosphocholine (AAGPC), or *a de novo* synthesis in which the last step is the transfer of a choline moiety from CDP-choline to alkylacetyl glycerol. The remodeling pathway involves two alternative mechanisms, both using PLA_2, for the generation of lyso-PAF, and a common final step by the transacetylation of lyso-PAF to PAF. AAGPC may be directly converted to lyso-PAF by the PLA_2-catalyzed removal of the *sn-2* acyl group. Alternatively, the acyl group of AAGPC may be transferred, utilizing a CoA-independent acyltransferase, to a "donor" lyso-plasmalogen (1-alk-1'-enyl-2-lyso-*sn*-glycero-3-phosphocholine) that is itself generated by the action of PLA_2 on plasmalogen. All of the enzyme activities known to be involved in PAF biosynthesis are present in brain (Lee et al., 1988; Goracci and Francesangeli, 1991; Blank et al., 1995). The contribution of each of these pathways to the biosynthesis of PAF under physiological or pathological conditions in brain has not yet been fully assessed, but the *de novo* route regulates steady-state levels of PAF, whereas the remodeling route is activated under stimulatory conditions. There is also some evidence that PAF acetyltransferase activity is also inducible via covalent modification of the enzyme, thought to be phosphorylation (Holland et al., 1992). In addition, none of the enzyme activities involved in the *de novo* route are known to be inducible, and there is even a report that *in vitro*, activity of the brain choline phosphotransferease is inhibited by elevated levels of free calcium (Francescangeli and Goracci, 1989). These findings suggest that the remodeling pathway is an important contributor to PAF production in brain.

PAF is inactivated through the cleavage of the *sn*-2 acetyl group by PAF-acetylhydrolases (PAF-AH). PAF-AHs are serine esterases with a substrate specificity for ether phospholipids possessing a short carbon chain in the *sn*-2 position. PAF-AH are a phospholipase A_2, but they do not share significant protein sequence homology with the typical intracellular or secretory PLA_2 beyond the Gly-Xaa-Ser-Xaa-Gly (where Xaa is any amino acid residue) motif common to many lipases (Tjoelker et al., 1995). There are two main classes of PAF-AH, plasma forms, consisting of a dimeric complex associated primarily with low-density lipoproteins (Stafforini et al., 1991), and various intracellular cytosolic forms of the enzyme activity (Hattori et al., 1993; Stafforini et al., 1993). Various other forms of PLA_2, and phospholipases C and D are also able to hydrolyze PAF *in vitro* (Snyder, 1995), but their contribution to the degradation of PAF *in vivo* is unknown. The brain-specific form of intracellular PAF-AH is a complex enzyme, consisting of two different subunits possessing catalytic activity and a third, presumably regulatory subunit (Hattori et al., 1993; Hattori et al., 1994a; Hattori et al., 1994b). The gene encoding this regulatory subunit has been found to correspond to the LIS-1 gene, a gene that is mutated in Miller-Dieker lissencephaly (Hattori et al., 1994a).

PAF acetylhydrolase can also hydrolyze species of oxidatively-damaged phospholipids that possess PAF-like activity. Free radical damage and fragmentation of the *sn-2* polyunsaturated fatty acid residue of some phosphatidylcholines yields species that have a short residual fatty acyl chain in the *sn-2* position. These molecules not only structurally resemble PAF, but have biological activity at the PAF receptor (Smiley et al., 1991; Zimmerman et al., 1994; Heery et al., 1995). Although these PAF-like species have not yet been studied in brain, they may well have a significant role in oxidative stress.

PAF Receptor

A PAF receptor has been cloned and characterized (Honda et al., 1991; Kunz et al., 1992; Sugimoto et al., 1992). Its predicted secondary amino structure identifies it as a member of the seven membrane-spanning domain, G protein-coupled receptor superfamily. The primary mode of signal transduction for this receptor is via a G protein-linked phospholipase C (Ali et al., 1994; Honda et al., 1995). PAF added to hippocampal neurons in culture elicits intracellular calcium fluxes, the dynamics of which are characteristic of release from intracellular stores rather than gating of extracellular calcium (Bito et al., 1992). However, additional signal transduction pathways have also been linked to the PAF receptor, most notably the mitogen-activated protein (MAP) kinase and associated protein kinase cascades (Honda et al., 1994; Kuruvilla et al., 1994; Mukerjee et al., 1998), and the down-regulation of cyclic AMP formation(Kester et al., 1992). Also, in some neuronal cell lines, PAF can raise intracellular calcium in a manner dependent upon the extracellular calcium concentration, implying the activation of cell-surface calcium channels (Kornecki and Ehrlich, 1988).

The PAF receptor gene structure displays two different transcription start sites contained in two distinct 5' non-coding exons (Mutoh et all, 1988). When alternatively spliced to a common exon containing the entire coding sequence, the result is the expression of two different transcripts (types 1 and 2) that contain the identical coding sequence, but differing in their 5' untranslated leader sequences. The leader sequences of types 1 and 2 both contain consensus binding sequences for the transcription factors NF-kappa-B and Sp-1, but type 2 alone contains additional binding sequences for AP-1 and AP-2. The transcripts show differential expression, with types 1 and 2 expressed in expressed in heart, spleen and kidney, and type 1 alone found in

peripheral leukocytes and brain.

PAF receptors have also been detected in the brain using ligand binding assays on cortical subcellular fractions (Marcheselli et al., 1990). These studies showed two different types of binding sites, one in synaptosomal membranes and another in microsomal membranes. The two sites differ in the kinetics of [^{3}H]-PAF binding; the microsomal membranes in fact display cold-ligand displacement kinetics characteristic of two binding sites.

The synaptosomal and microsomal sites also differ in their sensitivity to different types of PAF receptor antagonist, that in turn appear to influence different aspects of PAF action in the CNS. BN 52021, a terpenoid extracted from the leaf of the *Ginkgo biloba* tree, binds preferentially to the synaptosomal site whereas the synthetic hetrazepine BN 50730 shows complete specificity for the microsomal sites (Marcheselli et al., 1990; Marcheselli et al., 1994). The BN 52021-sensitive site appears to correspond to the cloned PAF receptor and to mediate the effects of PAF at the synapse. This antagonist selectively inhibits glutamate release from hippocampal neurons (Clark et al., 1992), perforant path LTP in hippocampal slices (Kato et al., 1994), and PAF-induced enhancement of memory tasks related to structures in the limbic system (Izquierdo et al., 1995) and striatum (Bazan et al., 1996). The BN 50730-sensitive site has yet to be isolated and there is no indication whether this represents an isoform of the cell-surface receptor, or a novel receptor. There is also no indication as yet as to which cell type(s) in the CNS express this form of PAF receptor. Thus far, the BN 50730-sensitive receptor has been linked with PAF-mediated effects on gene expression in the CNS. BN-50730, but not BN 52021, inhibits PAF- and neurotrauma-induced expression of the immediate-early genes *zif-268*, *c-fos*, and COX-2 (Marcheselli et al., 1994; Marcheselli et al., 1995). The inhibition by this antagonist

of COX-2 expression in a vasogenic cerebral edema model is accompanied by reduction of the blood-brain barrier breakdown (Marcheselli et al., 1995; Bazan et al., 1996). The selective effects of BN 50730 on PAF-induced gene expression are not limited to the brain or neuronal cells, as the drug also inhibits PAF-induced expression of immediate-early genes and selected metalloproteinase genes in a corneal epithelium organ culture system (HEP Bazan et al., 1993; Tao et al., 1994; Tao et al., 1995).

There are additional examples in neuronal and non-neuronal cells in which PAF selectively influences the expression of certain genes. PAF rapidly and transiently augments levels of the c-*fos* and c-*jun* transcription factor mRNAs in a neuronal cell line (Squinto et al., 1989). Phorbol esters and PAF synergistically stimulate c-*fos* expression suggesting that the transcriptional effects of PAF are not mediated by protein kinase C. PAF's effect is at the transcriptional level, as opposed to increasing the stability of the mRNA 5′ deletion mutagenesis studies of the c-*fos* promoter show that the calcium-response element is necessary for the PAF-induced response. There are other examples of PAF-induced gene expression in neuronal and non-neuronal cells including the heparin-binding epidermal growth factor in monocytes (Pan et al., 1995), *c-fos* and egr-2 in lymphoblastoid cell lines (Mazer et al., 1994), the transcription factor NF-kappa B and immunoglobulins in human B cell lines (Smith and Shearer, 1994), *c-fos* and zif/268 in rat astroglia (Dell'Albani et al., 1993), *c-fos* and TIS 1 in A-431 epidermoid carcinoma cells (Tripathi et al., 1991). In addition PAF is able to autoregulate gene expression of its own cell-surface receptor (Mutoh et al., 1994; Shirasaki et al., 1994).

Physiological significance of platelet-activating factor in glutamate neurotransmission, neuronal plasticity and memory.

PAF is an important modulator of glutamate neurotransmission, memory formation and neuronal plasticity. The following evidence supports this conclusion:

1) The PAF analog methylcarbamyl PAF (MC-PAF), but not biologically inactive lyso-PAF, when applied to synaptically connected hippocampal neurons, specifically augments endogenous excitatory synaptic responses, but not inhibitory GABA-mediated responses(Clark et al., 1992). It does not, however, augment the effects of exogenously-added glutamate, suggesting that this PAF effect is not at the level of glutamate receptors. The PAF receptor antagonist BN 52021 (at a concentration of 1μM) blocks the MC-PAF-enhanced neurotransmission. Since this antagonist is selective for the PAF receptor of synaptosomal membranes(Marcheselli et al., 1990), this implies that PAF under these conditions acts as a retrograde messenger on a presynaptic site. Incidentally, the neuroprotective effect of BN 52021 in ischemic-reperfusion in gerbil brain (Panetta et al., 1987) may be due partly to an inhibition of the PAF-induced release of excessive amounts of glutamate which attenuates excitotoxic damage.

2) PAF is a retrograde messenger in LTP. When applied to the CA1 subfield of the hippocampus, as in the hippocampal cell cultures described above, the bioactive lipid acts presynaptically via the cell-surface PAF receptor. Bath application of BN 52021 to hippocampal slices 25 minutes before or 10 minutes after stimulation blocks the sustained enhancement of excitatory postsynaptic potentials that follows tetanic stimulation of the Schaffer collateral pathway (Kato et al., 1994). This is not due to interference with NMDA receptor activity because the NMDA component of synaptic currents remains unaltered under all conditions. Thus

PAF must be acting downstream of the NMDA component of the LTP cascade. In the pretreated slices although an initial potentiation is seen, this declines to basal levels within 10 minutes, indicating that the effect of the antagonist is not on transmission of the initial stimulus. Indeed, there is a window of 5-10 minutes after commencement of stimulation in which BN52021 can still inhibit establishment of LTP. Very high concentrations of exogenous MC-PAF (40μM) can overcome this inhibition, indicating that the antagonist is acting directly on the presynaptic PAF receptor. Supporting this idea is the inability of the highly selective intracellular PAF receptor antagonist BN 50730 (Marcheselli and Bazan, 1994) to affect the establishment of LTP.

3) PAF enhances memory in rats performing an inhibitory avoidance task (Izquierdo et al., 1995) and in a water maze task (Bazan et al., 1996). In these instances specificity for the presynaptic PAF receptor was shown, as was neuroanatomical and temporal selectivity. For instance, in the inhibitory avoidance task PAF only had an effect when infused at specific times after training into defined areas of the limbic system, and in the water maze task PAF was only effective when infused into the striatum.

Arachidonic acid is another potential retograde messenger in hippocampal LTP (Williams et al., 1989) but slices require long-term exposure to high concentrations of the fatty acid. Furthermore inhibition of this form of LTP by the NMDA receptor antagonist D-2-amino-5-phosphopentanoic acid (APV) (O'Dell et al., 1991; Kato et al., 1991) indicates that arachidonic acid acts via a different mechanism than PAF, which acts independently of NMDA receptor. There is a report of PAF eliciting LTP which is sensitive to APV (Wieraszko et al, 1993). However, this was after prolonged exposure, and since arachidonic acid can potentiate NMDA responses (Miller et al., 1992) and PAF promotes the release of arachidonic acid (Kunievsky and

Yavin, 1992), then this form of LTP could arise from the effects of arachidonic acid.

PAF Activates Transcription of Response Genes

Several signals trigger the rapid and transient expression of immediate-early genes (primary genomic response genes, early response genes). In the brain physiological and pathological events (such as long-term potentiation, ischemia, seizures and NMDA receptor activation) initiate transcription of genes encoding transcription factors and thus have the potential to initiate cascades of gene expression. As a result of gene activation, long-term cellular responses such as neuronal plasticity occur.

PAF is a mediator of extracellular and intracellular signal/gene transcription coupling since it stimulates immediate-early gene expression in neuronal and other cells in culture. Moreover, selective PAF antagonists block gene transcription in transfected cells an *in vivo* in animal models of brain injury or seizures. PAF rapidly and transiently augments levels of the c-*fos* and c-*jun* transcription factor mRNAs in a neuronal cell line (Squinto et al., 1981). Phorbol esters and PAF synergistically stimulate c-*fos* expression suggesting that the transcriptional effects of PAF are not mediated by protein kinase C. The effect of PAF is at the transcriptional level, as opposed to increasing the stability of the mRNA 5′ deletion mutagenesis studies of the c-*fos* promoter show that the calcium-response element is necessary for the PAF-induced response. There are other examples of PAF-induced gene expression in neuronal and non-neuronal cells including the heparin-binding epidermal growth factor in monocytes (Pan et al., 1995), *c-fos* and egr-2 in lymphoblastoid cell lines (Mazer et al., 1994), the transcription factor NF-kappa B and immunoglobulins (Smith and Shearer, 1994) in human B cell lines, *c-fos* and

zif/268 in rat astroglia (Dell'Albani et al., 1993), and *c-fos* and TIS 1 in A-431 epidermoid carcinoma cells (Tripathi et al., 1991). PAF is also able to up-regulate gene expression of its own cell-surface receptor (Mutoh et al., 1994; Shirasaki et al., 1994). The intracellular PAF binding site antagonist BN 50730 blocks *c-fos* and zif/268 transcription factor expression induced in rat hippocampus and cerebral cortex by a single electroconvulsive shock (Marcheselli and Bazan, 1994), or zif/268 and inducible cyclooxygenase (PGHS-2, COX-2, TIS 10) in cryogenically-induced brain edema (Bazan et al., 1996).

PAF is involved in the seizure-induced accumulation of PGS-2 in the brain, and is thus linked to the metabolism of free arachidonic acid generated during seizures. PGS-2 transcriptional activity and protein expression is up-regulated in a single electrically-induced seizure (ECS) and in multiple, kainic acid (KA)-induced seizures, and PAF is involved in this mechanism. Furthermore, the induction of PGS-2, but not the transcription factor immediate-early gene zif-268, is more sensitive to the severe KA-induced seizures than ECS in a brain region, the hippocampus, selectively vulnerable to damage in this model. Induction of both genes by KA shows neuroanatomical specificity in the order hippocampus > cerebral cortex > striatum > brain stem > cerebellum. Nuclear run-on and Western blotting shows that both genes are transcriptionally activated, and that kainic acid up-regulation of prostaglandin G/H synthase-2 mRNA expression in hippocampus matches increased protein levels. Whereas the magnitude of hippocampal *zif*-268 mRNA induction is similar in both seizure models, peak induction of prostaglandin G/H synthase-2 mRNA is seven-fold greater in the kainic acid model than in the electroconvulsive shock model, and is much more prolonged. Pretreatment of animals by intracerebroventricular injection with the intracellular platelet-activating factor receptor

antagonist BN 50730 strongly attenuates kainic acid and electroconvulsive shock induction of prostaglandin G/H synthase-2 expression. The drug partially inhibits electroconvulsive shock induction of *zif*-268, but is relatively ineffective against kainic acid-induced *zif*-268 expression. Seizure-induced expression of both genes involves platelet-activating factor, but the mechanisms of induction must be otherwise distinct. The selectively elevated induction of hippocampal prostaglandin G/H synthase-2 by kainic acid correlates with a neuroanatomical region in which the agonist induces neuronal damage (Marcheselli and Bazan, 1991).

PAF Activates Expression of the Inducible Prostaglandin Synthase Gene

The rate-limiting step of prostaglandin synthesis is conversion of arachidonic acid to prostaglandin $_{He}$, catalyzed by prostaglandin G/H synthase. There are now known to be two forms of the enzyme, a "constitutive" enzyme (PGHS-1), and an "inducible" form (PGHS-2). The two enzymes have 61% amino acid sequence homology and share highly conserved domains essential for enzyme function, including glycosylation sites, heme binding sites, the aspirin acetylation sequence, and a region with homology to epidermal growth factor . They are, however, encoded by separate genes. PGHS-2 was first described as an immediate early gene induced by mitogens, cytokines, including by a number of mitogens and cytokines including serum, bFGF, TNFα, IL-1β, and $pp60^{v\text{-}src}$, and by phorbol esters, and down-regulated by glucocorticoids (Kujubu et al, 1991; O'Banion et al., 1991; Herschman, 1994). The rationale behind having two different forms of the enzyme is not entirely clear because, in cells which express both forms, up-regulation of PGHS-2 is not matched by an increase in prostaglandin synthesis. Nevertheless, recent findings suggest different subcellular localizations for the two enzymes: PGHS-1 is localized in the

endoplasmic reticulum, while PGHS-2 is enriched in the nuclear envelope. One line of thought is that PGHS-2 is responsible for the synthesis of PGs which act as intracellular signals, while PGHS-1 activity leads to the generation of PGs which act as extracellular mediators (Goetzl et al., 1995). PGHS-2 has been found in rat brain, where it is expressed throughout the forebrain in discrete populations and is enriched in the cerebral cortex and hippocampus (Yamagata et al., 1993). Its expression is rapidly and transiently induced by seizure or NMDA-dependent synaptic activity, while basal expression in the developing and adult appears to be regulated by synaptic activity. For instance, there is detectable expression in the developing animal from post-natal day 5 onwards, a period during which the rat brain is undergoing massive synaptic plasticity and developmental changes. The inducibilty of PGHS-2 expression in response to synaptic stimuli implies a role in neuronal plasticity (Yamagata et al., 1993).

Using constructs of the promoter of the murine inducible prostaglandin synthase gene transfected to neural and non-neural cell it was found that PAF stimulates expression of the luciferase reporter gene (Bazan et al, 1993a). Retinoic acid modulates gene expression through nuclear receptors that are members of a superfamily of ligand-dependent transcription factors. Since PAF may also affect gene expression through an intracellular site, the effect of retinoic acid was studied. Retinoic acid induces the expression of constructs of the TIS-10/PGS-2 promoter with a luciferase reporter transfected into neuroblastoma cells. Using the calcium phosphate co-precipitation transfection procedure in the presence of retinoic acid, there is a PAF-dependent (from 1 to 50 nM) activation of luciferase reporter constructs driven by regulatory regions of the TIS-10/PGHS-2 gene. The effect of PAF in indicating that a preexisting latent transcription factor(s) is engaged in the effect. Deletion studies of the TIS-10/PGHS-2

promoter/luciferase reporter constructs showed the deletion of sequences between -371 and -300 reduced the PAF inductive effect from 31-fold to 4.1-fold. BN 50730 inhibited the PAF inductive effect when incubated for 1 h with cultured cells before transfection. This indicates that a PAF receptor is involved in the expression of the inducible prostaglandin synthase and that a major PAF-responsive element lies within this region. Further deletions showed no response to added PAF.

Membrane-Derived Lipid Second Messengers in Neuronal Plasticity

The membrane-derived bioactive lipids regulate normal synaptic function (Bazan et al., 1993b) and plasticity responses and repair/regenerative responses. PAF is one of the messengers that links synaptic activation with transcription early response genes. Although PAF is most often referred to as a mediator of the inflammatory and immune responses as well as of cell injury, low PAF concentrations elicits sprouting in PC12 cells, whereas neuropathological changes occur when these cells are exposed to high PAF concentrations (Kornecki and Ehrlich, 1988). Therefore, PAF, when accumulated in large amounts, becomes a neuronal injury messenger (Bazan, 1997). This duality of PAF actions, both physiological and pathological, is also a feature of glutamate, an excitatory amino acid, that, by far, is the most abundant neurotransmitter of the mammalian brain. Glutamate plays a critical role in developmental plasticity and memory formation. However, in stroke, seizures, neurotrauma and some neurodegenerative diseases, there is an abnormally high synaptic accumulation of glutamate, making it a critical effector of excitotoxic damage. Other examples of neuronal mediators with this duality are interleukin-1 and, perhaps, amyloid peptide, that coexist with neural cells under

physiological conditions but may, when overproduced, be engaged in pathophysiology.

The presynaptic PAF binding site is linked to glutamate release (Clark et al., 1992) and involves PAF as a retrograde messenger in long-term potentiation (Kato et al., 1994). There is also a very high affinity binding site, localized intracellularly and linked to early response gene expression (Bazan et al., 1991). It is not yet known if two or more of these actions are interlinked or if there are different effects among the diverse cellular responses stimulated by PAF. The significance of the effect of PAF on gene expression may be related to long-term adaptive responses. PAF's ability to increase intracellular Ca^{2+} (Bito et al, 1992; Kornecki and Ehrlich, 1988), likely by activating a Ca^{2+} channel, by activating phospholipase C with resultant intracellular Ca^{2+} mobilization by inositol trisphosphate and by other actions is also a significative set of mechanisms through which synaptic plasticity may be affected.

PAF stimulation of the inducible prostaglandin synthase (COX-2) gene establishes another link with synaptic plasticity. The gene encodes the inducible COX-2, one of the enzymes that catalyzes the cyclooxygenation of arachidonic acid. The inducible prostaglandin synthase utilizes only a small proportion of the free arachidonic acid available under these conditions. PGE_2 synthesis may comprise a feedback loop of synaptic responses (**Figure 2**). The signal transduction pathways enabled by enhanced PLA_2 activation at the synapse provide powerful modulators of plasticity that potentially regulate memory formation and long term potentiation of the nervous system.

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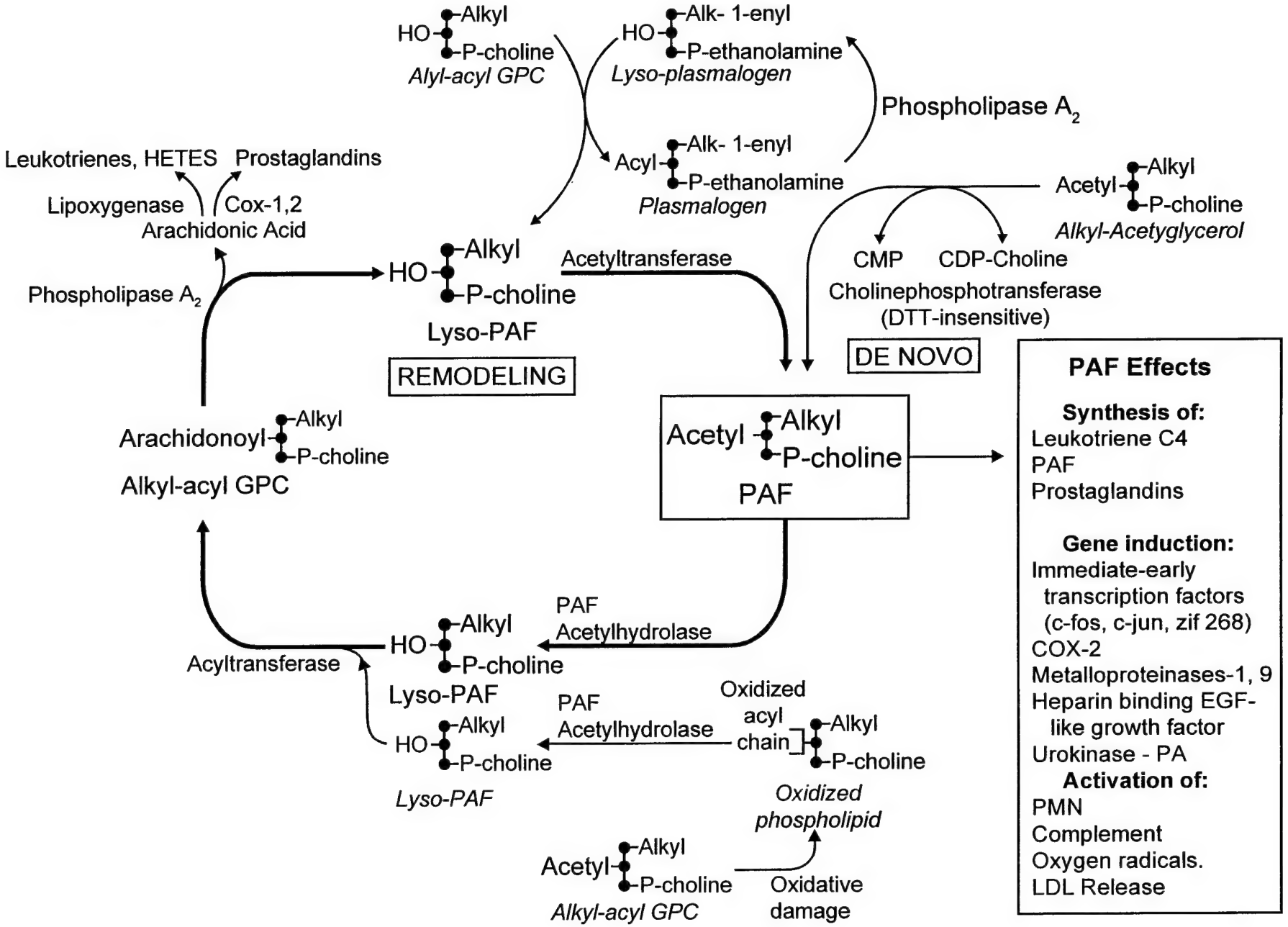
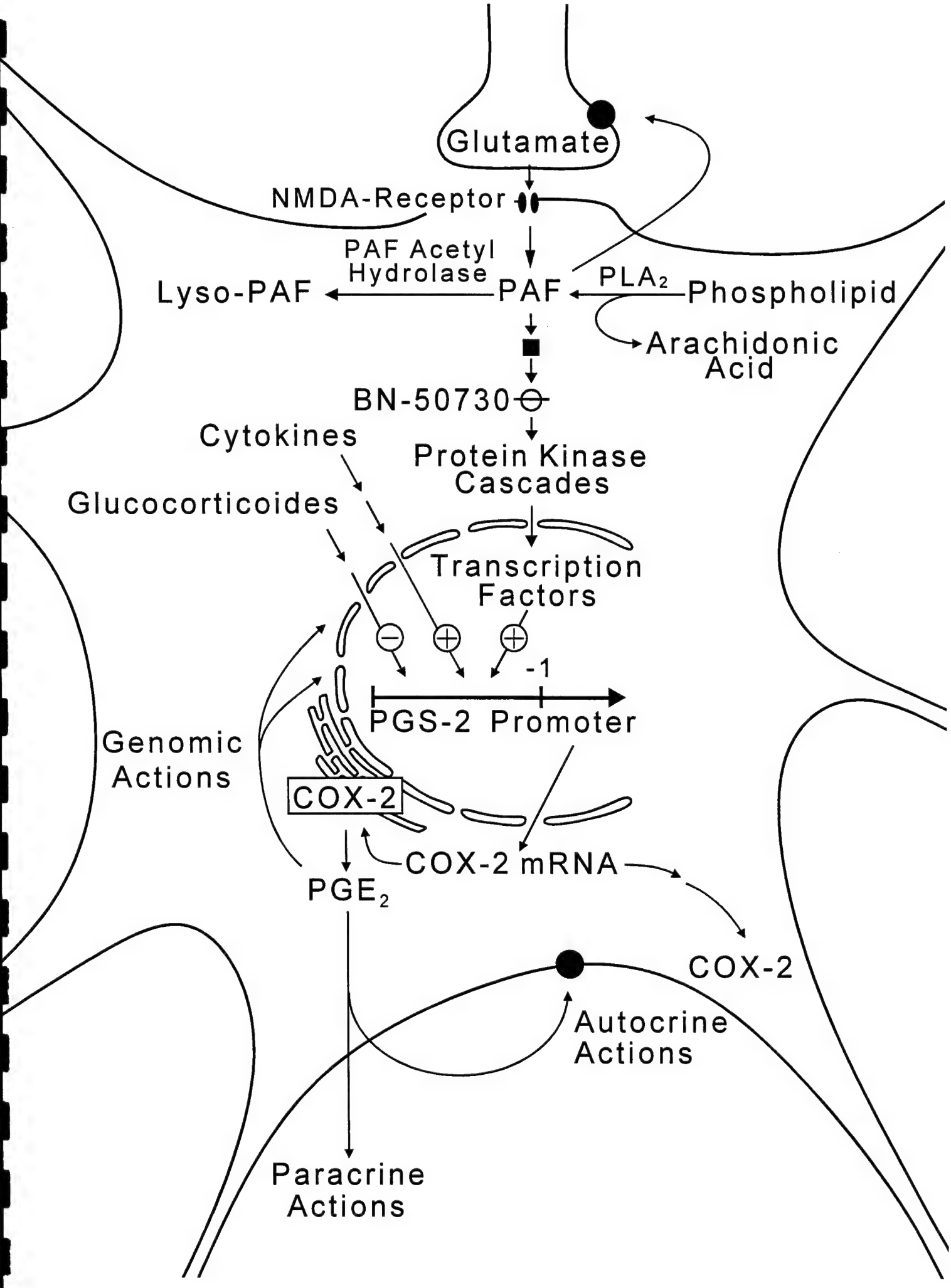
Figure legends.

Figure 1. Summary of pathways of PAF synthesis and degradation. The central portion of this figure represents the "PAF cycle" between the membrane phospholipid PAF precursor alkyl-acyl-GPC and biologically active PAF. The remodeling synthesis involves the production of lyso-PAF, generated from the PAF precursor alkyl-acyl GPC either by the direct action of phospholipase A_2, or the transfer of the *sn*-2 acyl chain to a "donor" lyso-plasmalogen, which is itself mobilized from membrane plasmalogen by phospholipase A_2 action. The de novo pathway involves the direct transfer of a choline moiety to alkyl-acetylglycerol. Note that PAF acetylhydrolase inactivates all PAF molecules regardless of their biosynthetic route, and additionally inactivates oxidatively damaged phospholipids that possess biological activity at the PAF receptor.

Figure 2. Excitatory synaptic transmission enhances the production of PAF, other messengers, and the expression of PGS-2. NMDA-receptor activation by glutamate leads to phospholipase A_2 activation and the generation of PAF and arachidonic acid. PAF is synthesized through other metabolic routes, as well. Other synaptic events, in addition to NMDA-receptor, may also be involved in the accumulation of PAF. PAF activates PGS-2 gene expression through a BN-50730-sensitive intracellular site, protein kinase cascades, and transcription factors. The PGS-2 promoter is also a target for cytokines (activation) and glucocorticoids (inhibition). PGS-2 protein (COX-2) then catalyzes the conversion of arachidonic acid into PGH_2, the precursor of eicosanoids. Constitutive PGS-1 also catalyzes this metabolic step (not shown). Prostaglandin

E_2 is depicted as a product of PGS-2 (COX-2). The specific products of over expressed PGS-2 are not yet known. PGE_2 is shown to potentially elicit genomic, paracrine, and/or autocrine effects.


HO
Alkyl
P-choline
Alyl-acyl GPC
HO
Alk- 1-enyl
P-ethanolamine
Lyso-plasmalogen
Phospholipase A2
Acyl
Alk- 1-enyl
P-ethanolamine
Plasmalogen
Leukotrienes, HETES
Prostaglandins
Lipoxygenase
Cox-1,2
Arachidonic Acid
Acetyl
Alkyl
P-choline
Alkyl-Acetyglycerol
CMP
CDP-Choline
Cholinephosphotransferase
(DTT-insensitive)
DE NOVO
Phospholipase A2
HO
Alkyl
P-choline
Lyso-PAF
REMODELING
Acetyltransferase
Arachidonoyl
Alkyl
P-choline
Alkyl-acyl GPC
Acetyl
Alkyl
P-choline
PAF
PAF Effects
Synthesis of:
Leukotriene C4
PAF
Prostaglandins
Gene induction:
Immediate-early transcription factors (c-fos, c-jun, zif 268)
COX-2
Metalloproteinases-1, 9
Heparin binding EGF-like growth factor
Urokinase - PA
Activation of:
PMN
Complement
Oxygen radicals.
LDL Release
PAF Acetylhydrolase
Acyltransferase
HO
Alkyl
P-choline
Lyso-PAF
PAF Acetylhydrolase
Oxidized acyl chain
Alkyl
P-choline
Oxidized phospholipid
HO
Alkyl
P-choline
Lyso-PAF
Acetyl
Alkyl
P-choline
Alkyl-acyl GPC
Oxidative damage


Glutamate
NMDA-Receptor
PAF Acetyl Hydrolase
Lyso-PAF
PAF
PLA2
Phospholipid
Arachidonic Acid
BN-50730
Cytokines
Glucocorticoides
Protein Kinase Cascades
Transcription Factors
-1
PGS-2 Promoter
Genomic Actions
COX-2
COX-2 mRNA
PGE2
COX-2
Autocrine Actions
Paracrine Actions

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Second Messengers, Long-Term Potentiation, Gene Expression and Epileptogenesis

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Running title: Second messengers, LTP, genes and epileptogenesis

Introduction

Various forms of brain damage triggers cellular and molecular events that often result in impaired synaptic plasticity that, in turn, induces recurrent seizures and epileptic discharges in the electroencephalogram. Hippocampal sclerosis, frequently the result of early neurotrauma or other forms of brain damage, is thought to be a significant event in epileptogenesis. Our hypothesis is that epileptogenesis involves a synaptic signaling dysfunction engaging lipid messengers, some of which modulate cell function. Synaptic messengers participate in synaptic strengthening, known also as long-term potentiation (LTP), a cellular form of memory formation. The persistence of changes in LTP indicates that synaptic plasticity has taken place and suggests that signal transduction includes modulation of gene expression. In fact, LTP induces several early response genes, including transcription factors, which, in turn, regulate the expression of gene cascades as well as of genes that encode proteins which participate in other functions such as extracellular matrix development and remodeling. Therefore perturbing synaptic signaling may contribute to aberrant synaptic plasticity and epileptogenesis. Once that abnormal connections are established, enhanced excitability promotes and contributes to seizure development and propagation. As a consequence, further seizures trigger additional damage. This vicious cycle of events in epileptogenesis has received experimental support (e.g., 1-6).

Phospholipases A_2, Arachidonic Acid and PAF

Phospholipid molecules of membranes from neurons and glial cells store a wide variety of lipid messengers. Receptor-mediated events and changes in intracellular [Ca^{2+}], as occurs during excitatory neurotransmission and in activity-dependent synaptic plasticity, modulate phospholipases that catalyze the release of bioactive moieties from phospholipids. These lipid messengers then

participate in intracellular and/or intercellular signaling. Bioactive lipids have significant neurobiological actions such as in the function of ion channels, receptors, neurotransmitter release, synaptic plasticity, and programs of neuronal gene expression. As a result, there is a rapid increase in the brain free fatty acid pool size, particularly of arachidonic acid (7, 8) and of the bioactive phospholipid, platelet-activating factor, (PAF, 1-0-alkyl-2-acetyl-sn-3-phosphocholine)(9, 10). Arachidonic acid is converted into a variety of biologically active derivatives through cyclooxygenation (11) and lipoxygenation (12), with these conversions being particularly enhanced during reperfusion or repeated seizures, when tissue oxygenation is coupled with an abnormally large free arachidonic acid pool. These early findings, as well as more recent ones (13) have suggested the involvement of the cytosolic, calcium dependent phospholipase A_2 ($cPLA_2$) in brain damage. This enzyme, which has a preference for arachidonoyl phospholipids, catalyzes the release from membranes of free arachidonic acid and of the PAF precursor, lyso PAF. The importance of $cPLA_2$ in brain injury is strongly supported by the recent finding that $cPLA_2$ knockout mice have substantially reduced infarcts and lesser neurological deficits following transient middle cerebral artery occlusion (14). While these observations do not demonstrate that $cPLA_2$ is directly involved in epileptogenesis, several ongoing studies are addressing this question.

Brain damage and seizures disrupt the tightly regulated events that control the production and accumulation of lipid messengers, such as free arachidonic acid, diacylglycerol and PAF under physiological conditions. Rapid activation of phospholipases, specifically of phospholipase A_2 (PLA_2) as it occurs at the onset of injury and seizures (7, 8), was predicted to play a central role in excitable membrane function and pathology (7). There are a wide variety of PLA_2s (15), and current investigations aim to define those involved in synaptic events. In addition to the role(s) of

intracellular PLA_2s in lipid messenger formation, it has recently been discovered that a low molecular weight, secretory PLA_2 synergizes glutamate-induced neuronal damage (16). Whereas pathways leading to PLA_2 activation/release are part of normal neuronal function, seizures and injury enhance these events, overproducing PLA_2-derived lipid messengers, (e.g. enzymatically produced arachidonic acid oxygenation metabolites and non-enzymatically generated lipid peroxidation products), involved in neuronal damage. Among the consequences of PLA_2 activation by seizures are alterations in mitochondrial function by the rapid increase in the brain free fatty acid pool size (e.g. uncoupling of oxidative phosphorylation from respiratory chain) (17, 18), the generation of lipid messengers and also by impairments in mitochondrial function that, in turn, lead to enhanced release of reactive oxygen species to the cytoplasm.

PAF is a very potent and short-lived lipid messenger. It is known to have a wide range of actions: as a mediator of inflammatory and immune responses, as a second messenger, and as an inducer of gene expression. Thus, in addition to its acute roles, PAF can potentially participate in long-term modifications of cellular physiology and brain function. In this article, the significance of the messenger PAF in synaptic function, including LTP, and neuronal gene expression relevant to epileptogenesis is discussed.

PAF enhances excitatory synaptic transmission and modulates synaptic plasticity.

PAF enhances glutamate release in synaptically-paired rat hippocampal neurons in culture (19). The PAF analog methylcarbamyl (mc-PAF), but not biologically inactive lyso PAF, increases excitatory synaptic responses. The inhibitory neurotransmitter γ-aminobutyric acid is unaffected by mc-PAF under these conditions. The presynaptic PAF receptor antagonist BN 52021 (see below)

blocks the mc-PAF-enhanced glutamate release. In addition, mc-PAF increases presynaptic glutamate release, since it does not augment the effects of exogenously added glutamate, and it evokes spontaneous synaptic responses characteristic of enhanced neurotransmitter release. Therefore, as a modulator of glutamate release, PAF participates in long-term potentiation (20, 21), synaptic plasticity and memory formation.

PAF, a neuronal injury messenger, contributes to excitotoxicity by enhancing glutamate release.

Seizures and ischemia increase PAF content in brain (9, 10). Furthermore, brain is endowed with a variety of degradative enzymes that rapidly convert PAF to biologically inactive lyso PAF (22-24). Presynaptic membranes display PAF binding that can be displaced by BN 52021, a terpenoid extracted from the leaf of the *Ginkgo biloba* tree, which binds preferentially to the synaptosomal site (25). It is likely that this PAF binding site is the seven transmembrane PAF receptor (26-28). BN 52021 inhibits both PAF-induced glutamate release (19) and long-term potentiation (20-21). Moreover, this antagonist is neuroprotective in ischemia-reperfusion damage in the gerbil brain (29). Taking these finding together, PAF, when overproduced at the synapse during seizures, will promote enhanced glutamate release that in turn, through the activation of post-synaptic receptors, will contribute to enhanced excitotoxicity.

PAF is a transcriptional activator of the early response gene, prostaglandin endoperoxide synthase-2 (COX-2).

In addition to its modulatory effect on synaptic transmission and neural plasticity, PAF activates receptor-mediated immediate early gene expression (30-33). Since PAF is a phospholipid

and can pass through membranes, it is rapidly taken up by cells. An intracellular binding site with very high affinity, yet pharmacologically distinct from the presynaptic site, was found in brain (25). The synthetic hetrazepine BN 50730 is selective for this intracellular site and blocks PAF-induced gene expression of the inducible form of prostaglandin G synthase in transfected cells (34).

Prostaglandin G/H synthase-2 (PGS-2, COX-2, TIS-10) catalyzes the cyclooxygenation and peroxidation of arachidonic acid into PGH_2, the precursor of biologically active prostaglandins, thromboxanes and prostacyclin (35-36). COX-1 also catalyzes the same first committed step of the arachidonic acid cascade. COX-2, however, is expressed in response to mitogenic and inflammatory stimuli and is encoded by an early-response gene. In contrast, COX-1 expression is not subject to short-term regulation. Neurons in the hippocampus, as well as in a few other brain regions, are unlike other cells in that they display basal levels of COX-2 expression (37). This expression is modulated by synaptic activity, LTP and involves the N-methyl-D-aspartate class of glutamate receptors (37, 38).

PAF is a transcriptional activator of COX-2, as PAF induces mouse COX-2 promoter-driven luciferase activity transfected in neuroblastoma cells (NG108-15 or SH-SY5Y) and in NIH 3T3 cells. The intracellular PAF antagonist, BN 50730, inhibits PAF activation of this construct (34). Figure 1 outlines the role of PAF as a presynaptic messenger.

Sustained transcriptional upregulation of COX-2 during kainic acid-induced seizures in hippocampus.

The abundance in brain of several early-response gene transcripts shows rapid and transient increases during cerebral ischemia and after seizures (32). Several early-response genes encode

transcription factors which in turn modulate the expression of other genes, whereas others encode inducible enzymes. The glutamate analog, kainic acid, when systemically injected in rats, promotes extensive neuronal damage, and a status epilepticus-like condition (particularly in the hippocampus). It also induces early-response genes such as the transcription factor *zif*-268. COX-2 is also induced under these conditions, but there are striking differences in the magnitude and duration of the induction of COX-2 as compared with *zif*-268 (Fig 2). Kainic acid-induced upregulation of COX-2 and *zif*-268 transcription in hippocampus differed , as did the time course of expression following a single seizure triggered by electroconvulsive shock (39). In both conditions, the peak of COX-2 mRNA abundance was later than *zif*-268. In kainic acid treated animals, COX-2 reached a 71-fold increase over controls in 3 hours, whereas *zif*-268 peaked at 1 hr with a 10-fold increase. COX-2 mRNA, 2 hr after kainic acid injection, showed a 35-fold increase in hippocampus as compared with only a 5.5 fold increase in *zif*-268 (39). Also COX-2 peak in mRNA abundance was evident at 3 hrs (71-fold increase) as compared with 1 hr for *zif*-268 (10-fold increase). *Zif*-268 mRNA time-course of changes in the hippocampus corresponds to the expected profile of early-response genes, i.e., a rapid decrease in abundance after the peak. COX-2 on the other hand, displayed sustained upregulation for several hours after kainic acid injection (5.2 fold increase at 12 hours) (39).

Possible significance of the platelet activating factor-COX-2 intracellular signaling pathway in epileptogenesis.

A PAF-stimulated signal transduction pathway is a major component of the kainic acid-induced COX-2 expression in hippocampus. This conclusion is based upon the finding that a) PAF induces mouse COX-2 promoter-driven luciferase activity in transfected cells, and BN 50730

inhibits this effect (34); and b) BN 50730 (given intracerebroventricularly 15 min prior to kainic acid) inhibits kainic acid-induced COX-2 mRNA accumulation in hippocampus by 90% (39). Both PAF (23) and COX-2 (40) are potent mediators of the injury/inflammatory response. PAF (19-21) and COX-2 (37, 38) are also interrelated in neuronal plasticity. The PAF transcriptional activation of COX-2 may provide clues about novel neuronal cell death pathways. The antagonist BN 50730 was much less effective against *zif*-268 expression. In fact, the delayed hippocampal induction of COX-2 by kainic acid precedes selective neuronal apoptosis by this agonist in this neuroanatomical region (41).

In epilepsy, the significance of the PLA_2-related signaling triggered by seizures or injury may be part of events finely balanced between neuroprotection and neuronal cell death. The precise events that would tilt this balance toward the latter are currently being explored. It is interesting to note that PAF, being short-lived and rapidly degraded by PAF acetylhydrolase (42), is a long-term signal with consequences to neurons though COX-2 sustained expression. COX-2 is localized in the nuclear envelope and perinuclear endoplasmic reticulum. The overexpression of hippocampal COX-2 during cerebral ischemia and seizures may in turn lead to the formation of neurotoxic metabolites (e.g. superoxide). Current investigations aim to determine whether or not other messengers cooperate to enhance neuronal damage (e.g. nitric oxide) and the possible involvement of astrocytes and microglial cells. Further understanding of these potentially neurotoxic events involving lipid messengers and COX-2 will permit the identification of new therapeutic strategies for the management of epilepsy.

Acknowledgments

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Figure 1: **Seizure-triggered signaling events linking synapse activation and COX-2 gene expression in neurons.** NMDA-receptor activation by glutamate leads to phospholipase A_2 activation and the generation of PAF and of arachidonic acid. PAF is synthesized through other metabolic routes as well (19). PAF activates COX-2 gene expression through a BN-50730-sensitive intracellular site, protein kinase cascades and transcription factors. The COX-2 promoter is also a target for cytokines (activation) and glucocorticoids (inhibition). COX-2 protein then catalyzes the conversion of arachidonic acid into PGH_2, the precursor of eicosanoids. Constitutive COX-1 also catalyzes this metabolic step (modified from N. Bazan, *Primer Cerebrovascular Diseases*. M Welsh, L Chaplan, D Reis, B Siesjö, B Weir (eds), Academic Press, 1997, with permission).

Figure 2. **COX-2 and *zif*-268 during kainic acid-induced seizures: Time course of changes in relative mRNA abundance and transcriptional activity in hippocampus.**

(A) Relative abundance of COX-2 and *zif*-268 mRNAs compared to GAPDH mRNA, as assessed by northern analysis. (n=9-12 for each time point from 3 separate experiments, error bars ± 1 s.d.). (B) Transcriptional activity of COX-2, COX-1 and *zif*-268 genes assessed by nuclear run-on transcription. (n=3-4 from 3 separate experiments). Data are normalized to transcriptional activity of GAPDH (modified from Marcheselli and Bazan, *J. Biol. Chem*, 1996, with permission).

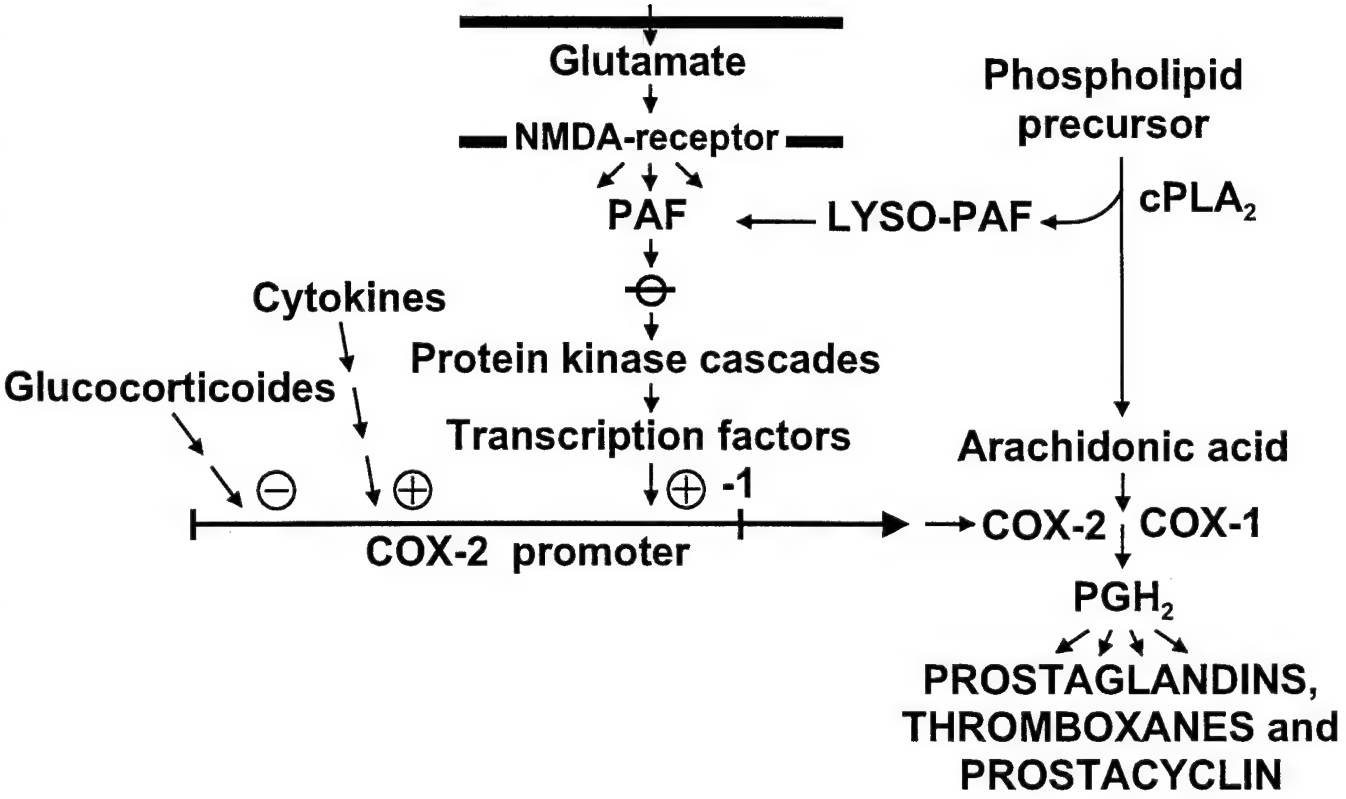
Figure 3. **Inhibition by intracerebroventricular injection of kainic acid-induced COX-2 protein accumulation (open bars), but not of COX-1 (closed bars) in rat hippocampus.**

(A) Representative Western blots. Animals were pretreated intracerebroventricularly with BN-50730 in DMSO (experimental) or DMSO alone (control) 15 min before KA injection. Samples were

collected 6 hours after KA treatment.

(B) Quantification of Western blot data expressed as a percent increase over control (vehicle-pretreated) values. (n=10-12 from 3 separate experiments, error bars ± 1 s. d.) (from Marcheselli and Bazan, *J. Biol. Chem*, 1996, with permission)

Figure 4. **Potential sites of drug action on the PAF - COX-2 axis.** Excitatory synaptic transmission enhances the production of PAF and of other messengers. PAF activates transcription of COX-2. COX-2 protein is located in the perinuclear region. It is not known the nature of the eicosanoids generated. PGE_2 is depicted as a product that in turn may elicit on the nuclear, paracrine and autocrine actions. Three possible drug targets are indicated as 1, 2 or 3.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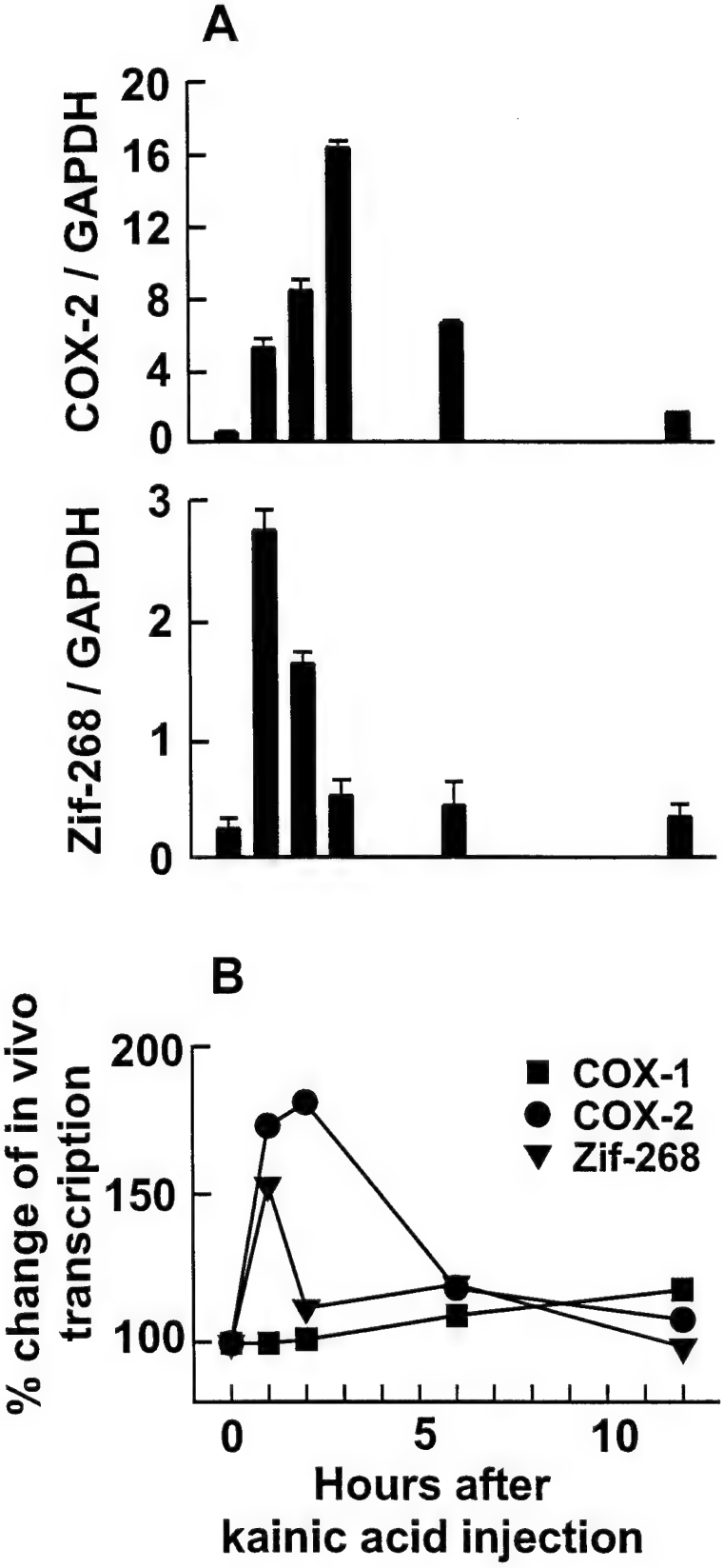
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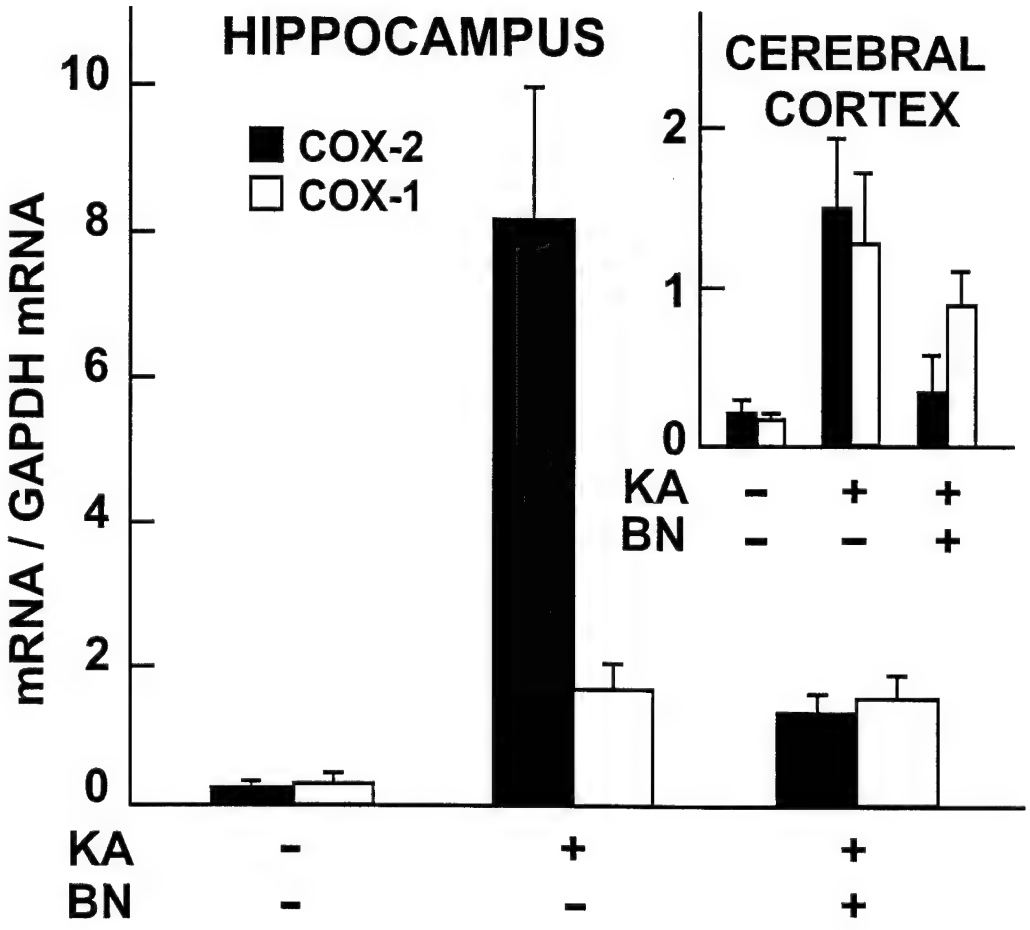
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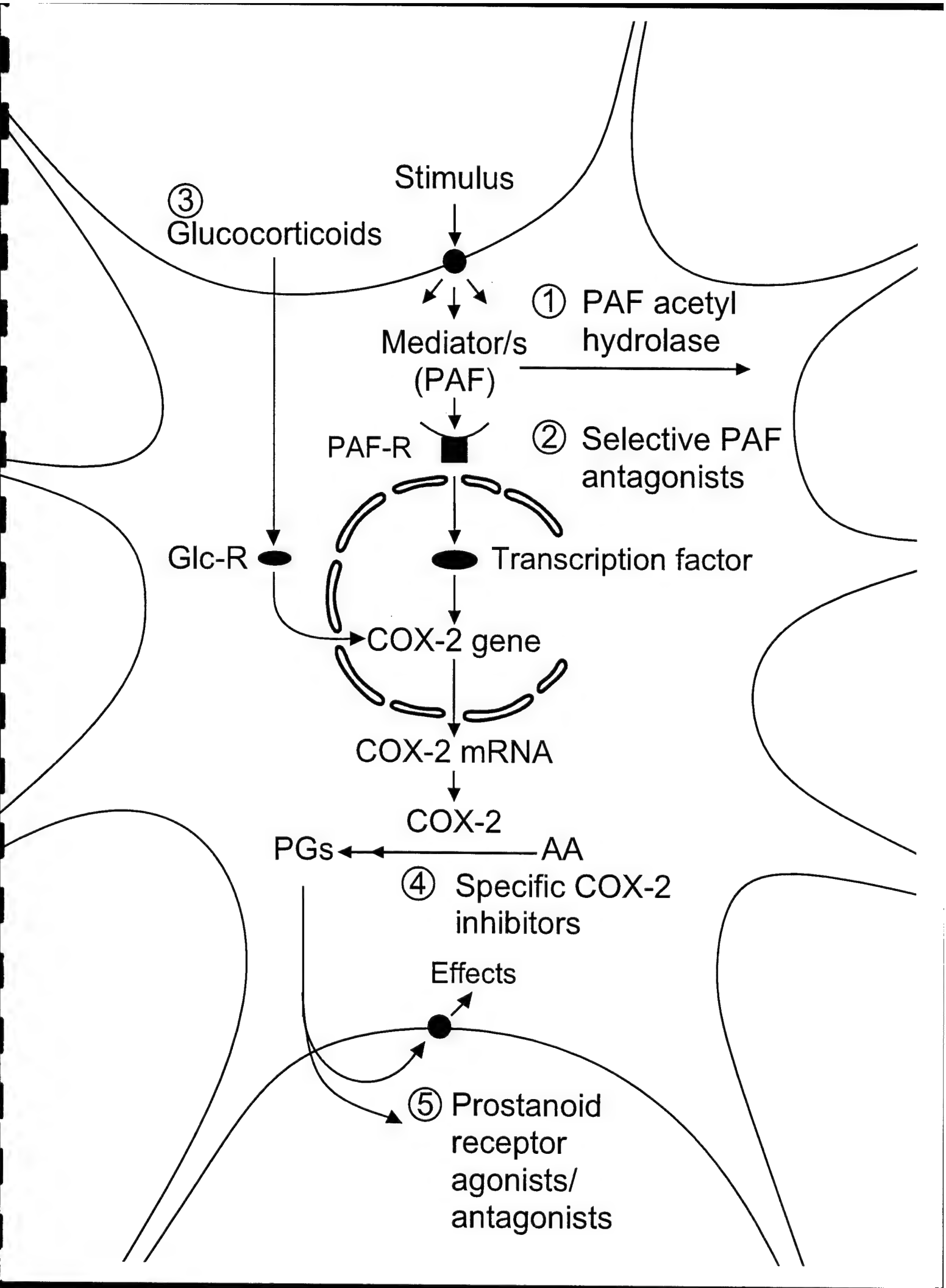
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COX-2 mRNA
COX-2
PGs
AA
④ Specific COX-2 inhibitors
Effects
⑤ Prostanoid receptor agonists/ antagonists

The Neuromessenger Platelet Activating Factor in Plasticity and Neurodegeneration

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Abstract

Synaptic activation leads to the formation of arachidonic acid, platelet-activating factor (PAF, 1-O-alkyl-2-acyl-sn-3-phosphocholine)and other lipid messengers. PAF is a potent bioactive phospholipid in synaptic plasticity. PAF enhances presynaptic glutamate release, is a retrograde messenger in long-term potentiation and enhances memory formation. PAF also couples synaptic events with gene expression by stimulating a FOS/JUN/AP-1 transcriptional signaling system, as well as transcription of COX-2 (inducible prostaglandin synthase.) Since the COX-2 gene is also involved in synaptic plasticity, the PAF-COX-2 pathway may have physiological significance. Seizures, ischemia and other forms of brain injury promote phospholipase A_2 (PLA_2) over activation, resulting in the accumulation of bioactive lipids at the synapse. PAF, under these pathological conditions, behaves as a neuronal injury messenger by at least two mechanisms: a) enhancing glutamate release and, b) by sustained augmentation of COX-2 transcription. These events link PAF with neurodegeneration. The upstream intracellular pathways of signal transduction involved in neuronal or photoreceptor cell apoptosis are not well understood and certainly may involve stress sensitive kinases. PAF is a transcriptional activator of the COX-2 gene. BN 50730, a potent intracellular PAF antagonist, blocks COX-2 induction. COX-2 transcription and protein expression are upregulated in the hippocampus in kainic acid induced epileptogenesis. There is a selectively elevated induction of COX-2 (72-fold) by kainic acid preceding neuronal cell death. BN 50730 administered by icv injection blocks seizure-induced COX-2 induction.

Introduction

In neuronal and glial cell signaling, several diffusible messengers other than nitric oxide (NO) are active participants, e.g., PAF, prostaglandins and lipoxygenase products are prominent examples. These bioactive lipids share the following: a) they are stored in the structure of membrane phospholipids; b) synaptic activation promotes their release; c) phospholipase A_2 (PLA_2) activation catalyzes the initiation of the pathway leading to their synthesis; and, d) seizures and ischemia activate their release. Prostaglandins (1) and lipoxygenase reactions products (2) are synthetized by cyclooxygenases and lipoxygenases and rapidly accumulate in brain during stimulation or in ischemia. These biologically active metabolites are released upon depolarization from neural tissue (3, 4). 12-hydroxyeicosatrienoic (12-HETE) is a prominent diffusable arachidonic acid metabolite in Aplysia Californica neurons (5, 6), during K^+-induced depolarization in the retina (3), as well as in synaptosomes from brains of rats undergoing bicuculline induced seizures (7). 12-HETE in Aplysia Californica has been implicated in the modulation of synaptic transmission through ion channels and in behavior (5, 6). PLA_2 activation is an early event in the brain response to injury, such as ischemia or at the onset of seizures (8). Endogenous free arachidonic acid accumulates in synaptosomes from rats undergoing seizures (7), strongly suggesting a synaptic location for the phospholipase A_2 activated during brain stimulation.

Although the oxygenated metabolites of arachidonic acid (e.g., prostaglandins and

lipoxygenase reaction products) have been found to change during stimulation and injury, only recently defined sites of action and their potential significance has been uncovered.

The cloning of prostaglandin receptors and the finding that adenylcylcylase activation follows prostaglandin receptor occupancy has defined a signaling route whereby the diffusable prostaglandins modulate protein kinase A and the transcription factor, CREB (cyclic response element binding protein), leading to the control of gene expression (9). Moreover, it has recently been found that prostaglandins modulate glutamate release from glial cells (10) suggesting that intercellular prostaglandins (derived, perhaps, from the activation of certain neurons) may modulate excitatory synaptic transmission.

Since the diffusible lipid mediators, prostaglandins, PAF and others are generated by PLA_2 activation, it is important to highlight the significance of this enzyme (Fig 1). First, there are several PLA_2's: cytosolic ($cPLA_2$), secretory ($sPLA_2$) and calcium-independent ($iPLA_2$). Limitations of the present chapter do not permit and in-depth discussion of each form. However, their relevance to brain function and, particularly, in cerebrovascular diseases was recently reviewed (11). Some highlights follow. $sPLA_2$ is released along with neurotransmitters (12) and strongly potentiates glutamate excitotoxicity (13). $cPLA_2$ may be critical in spreading depression through the generation of PAF (14). The initiating event for the synthesis of several of the diffusible lipid messengers seems to be $cPLA_2$. This enzyme is activated by the NMDA-mediated

rise in intracellular calcium and is further enhanced by ERK phosphorylation. $cPLA_2$ has a preference for arachidonoyl phospholipids. The important function of $cPLA_2$ in brain stimulation is supported by the recent finding that $cPLA_2$ knockout mice have substantially reduced infarcts and neurological deficits following transient middle cerebral artery occlusion (10). This strongly implicates $cPLA_2$ in postischemic brain injury, as previously suggested for a PLA_2 (8, 15).

The plenary lecture at this symposium on diffusible messengers will focus on the physiological significance and pathological role of the neuromessenger, PAF, closely related by its origins to the other lipids messengers briefly described above.

PAF enhances presynaptic glutamate release and modulates plasticity.

PAF is one of the most potent bioactive lipids known. Figure 2 outlines the metabolic pathways for its synthesis and degradation. PAF is implicated in the inflammatory/injury response (16), as well as in physiological processes (17). PAF enhances glutamate release in synaptically-paired rat hippocampal neurons in culture (18). The PAF analog methylcarbamyl (mc-PAF), but not biologically inactive lyso PAF, increases excitatory synaptic responses. The inhibitory neurotransmitter γ-aminobutyric acid is unaffected by mc-PAF under these conditions. The presynaptic PAF receptor antagonist BN 52021, a terpenoid extracted from the leaf of the *Ginkgo biloba* tree which binds preferentially to the synaptosomal PAF binding site (19), blocks the mc-PAF enhancement of glutamatergic neurotransmitter release. PAF enhancement of

glutamatergic synaptic function has been shown to endure for periods relevant to the long-term potentiation of neurotransmission thought to underlie memory formation (20). Indeed, *in vivo* administration of PAF has been shown to disrupt memory (21). PAF has been shown to enhance memory in rats performing an inhibitory avoidance task (22) and in a water maze task (21). In these instances, specificity for the synaptic PAF receptor was shown, as was neuroanatomical and temporal selectivity. For instance, in the inhibitory avoidance task, PAF only had an effect when infused at specific times after training into defined areas of the limbic system, and, in the water maze task, PAF was only effective when infused into the striatum. Therefore, as a modulator of glutamate release, PAF participates in long-term potentiation (LTP), synaptic plasticity and memory formation. Figure 3 represents the presynaptic and postsynaptic regulatory sites involving PAF.

Activation of Synaptic Phospholipases A_2 During Seizures

One of the earliest consequence of seizures is cleavage of phospholipids in excitable membranes. Phospholipid molecules of membranes from neurons and glial cells store a wide variety of lipid messengers. Receptor-mediated events and changes in intracellular [Ca^{2+}], as occurs during excitatory neurotransmission and in activity-dependent synaptic plasticity, activates phospholipases that catalyze the release of bioactive moieties from phospholipids. These messengers then participate in intracellular and/or intercellular signaling pathways. Bioactive lipids have significant

neurobiological actions in neurotransmitter release, synaptic plasticity, and programs of neuronal gene expression. Accordingly, contemporary research into bioactive lipids has focused on their neurobiological significance and a major area to be developed is epileptic brain damage and epileptogenesis.

Seizures disrupt the tightly regulated events that control the production and accumulation of lipid messengers, such as free arachidonic acid, diacylglycerol and PAF under physiological conditions. Rapid activation of phospholipases, particularly of PLA_2, occurs at the onset of seizures (23). There are a wide variety of PLA_2s (24), and current investigations aim to define those affected by epileptic seizures. For example, in addition to the role(s) of intracellular PLA_2s in lipid messenger formation, it has recently been discovered that a low molecular weight, secretory PLA_2 similar to that released from astrocytes (25) and neurons (12) has been recently shown to synergize glutamate-induced neuronal damage (13). Whereas pathways leading to PLA_2 activation/release are part of normal neuronal function, seizures enhance these events, overproducing PLA_2-derived lipid messengers, (e.g. enzymatically produced arachidonic acid oxygenation metabolites, non-enzymatically generated lipid peroxidation products and other reactive oxygen species), involved in neuronal damage. Among the consequences of PLA_2 activation by seizures are alterations in mitochondrial function by the rapid increase in the brain free fatty acid pool size (e.g. uncoupling of oxidative phosphorylation from respiratory chain) (26, 27) and the generation of lipid messengers. Arachidonic acid and its prostaglandin metabolites may also acutely distort signal

transduction by altering glutamate receptor affinity (23, 28) and sphingomyelinase enzyme activity (29). Prostaglandins are also known to regulate transcription of immediate early genes (30), possibly by binding to nuclear transcription factor receptors (31, 32).

PAF is a very potent and short-lived lipid messenger. It is known to have a wide range of actions: as a mediator of inflammatory and immune responses, as a second messenger, and as a potent inducer of gene expression. Thus, in addition to its acute roles, PAF can potentially mediate longer-term effects on cellular physiology and brain function. In this article, the significance of PAF in synaptic function and neuronal gene expression relevant to epilepsy is discussed.

PAF, a neuronal injury messenger, contributes to excitotoxicity by enhancing glutamate release.

Seizures and ischemia increase PAF content in brain (33). Furthermore, brain is endowed with a variety of degradative enzymes that rapidly convert PAF to biologically inactive lyso PAF (17, 34). Presynaptic membranes display PAF binding that can be displaced by BN 52021 (31). It is likely that this PAF binding site is the seven transmembrane PAF receptor that has been cloned by T. Shimizu, et al (for reference see 23). BN 52021 inhibits both PAF-induced glutamate release (18) and long-term potentiation (20). Moreover, this antagonist is neuroprotective in ischemia-reperfusion damage in the gerbil brain (for reference see 23). Taking these finding together, PAF,

when overproduced at the synapse during seizures, will promote enhanced glutamate release that in turn, through the activation of post-synaptic receptors, will contribute to excitotoxicity.

PAF is a transcriptional activator of prostaglandin endoperoxide synthase-2

In addition to its modulatory effect on synaptic transmission and neural plasticity, PAF activates receptor-mediated immediate early gene expression (35, 36). Since PAF is a phospholipid and can pass through membranes, it is rapidly taken up by cells. An intracellular binding site with very high affinity, yet pharmacologically distinct from the presynaptic site, was found in brain (19). The synthetic hetrazepine, BN 50730, is selective for this intracellular site and blocks PAF-induced gene expression of the inducible form of prostaglandin G synthase in transfected cells (37).

Prostaglandin G/H synthase-2 (PGS-2, COX-2, TIS-10) catalyzes the cyclooxygenation and peroxidation of arachidonic acid into PGH_2, the precursor of biologically active prostaglandins, thromboxanes and prostacyclin. COX-1 also catalyzes the same first committed step of the arachidonic acid cascade. COX-2, however, is expressed in response to mitogenic and inflammatory stimuli and is encoded by an early-response gene. In contrast, COX-1 expression is not subject to short-term regulation. Neurons in the hippocampus, as well as in a few other brain regions, are unlike other cells in that they display basal levels of COX-2 expression (38). This expression is modulated by synaptic activity, LTP and involves the N-methyl-D-aspartate class of

glutamate receptors (38, 39).

PAF is a transcriptional activator of COX-2, as PAF induces mouse COX-2 promoter-driven luciferase activity transfected in neuroblastoma cells (NG108-15 or SH-SY5Y) and in NIH 3T3 cells. The intracellular PAF antagonist, BN 50730, inhibits PAF activation of this construct (37). Figure 1 outlines the role of PAF as a presynaptic messenger.

Sustained transcriptional upregulation of COX-2 during kainic acid-induced seizures in hippocampus.

The abundance in brain of several early-response gene transcripts shows rapid and transient increases during cerebral ischemia and after seizures. Several early-response genes encode transcription factors which in turn modulate the expression of other genes, whereas others encode inducible enzymes. The glutamate analog, kainic acid, promotes extensive neuronal damage, particularly in the hippocampus, and also induces early-response genes such as the transcription factor *zif*-268. COX-2 is also induced under these conditions, but there are striking differences in the magnitude and duration of the induction of COX-2 as compared with *zif*-268 (Figs 4 and 5). COX-2 mRNA, 2 hr after kainic acid injection, showed a 35-fold increase in hippocampus as compared with only a 5.5 fold increase in *zif*-268 (40). Also COX-2 peak in mRNA abundance was evident at 3 hrs (71-fold increase) as compared with 1 hr for *zif*-268 (10-fold increase). *Zif*-268 mRNA time-course of changes in the hippocampus corresponds to

the expected profile of early-response genes, i.e., a rapid decrease in abundance after the peak. COX-2 on the other hand, displayed sustained upregulation for several hours after kainic acid injection (5.2 fold increase at 12 hours) (40) (Fig 6).

A sustained upregulation of COX-2 mRNA also follows KCl-induced spreading depression in brain slices, a model of the depolarization-mediated neurotoxicity that contributes to penumbral brain damage following focal ischemia (14). As in the kainic acid model, the peak in neocortical COX-2 mRNA abundance occurred at 4 hours post-treatment and returned to baseline only after 24 hours. This upregulation was abolished by the NMDA glutamate receptor antagonist MK-801. In an *in vivo* ischemic model of penumbral neurotoxicity, as sustained induction of COX-2 mRNA was also attenuated by MK-801, and by inhibiting PLA_2 activity (14).

The platelet activating factor-prostaglandin G synthase-2 intracellular signaling pathway in epileptogenesis

A PAF-stimulated signal transduction pathway is a major component of the kainic acid-induced COX-2 expression in hippocampus. This conclusion is based upon the finding that a) PAF induces mouse COX-2 promoter-driven luciferase activity in transfected cells, and BN 50730 inhibits this effect (19); and b) BN 50730 (given intracerebroventricularly 15 min prior to kainic acid) inhibits kainic acid-induced COX-2 mRNA accumulation in hippocampus by 90% (35) (Fig 7). Both PAF (17) and COX-2

(41) are potent mediators of the injury/inflammatory response. PAF (13) and COX-2 (23, 39) are also interrelated in neuronal plasticity. The PAF transcriptional activation of COX-2 may provide clues about novel neuronal cell death pathways. The antagonist BN 50730 was much less effective against *zif*-268 expression. In fact, the delayed hippocampal induction of COX-2 by kainic acid precedes selective neuronal apoptosis by this agonist in this neuroanatomical region. Furthermore, recent experiments demonstrate that the COX-2 specific antagonist NS-398, but not the COX-1 selective antagonist valeryl salicylate, attenuates the NMDA-induced neuronal death in primary cortical cell cultures (42). Figure 8 outlines how the signaling events evolving from the synapse may modulate COX-2 expression.

For example, the significance of the PLA_2-related signaling triggered by ischemia reperfusion may be part of events finely balanced between neuroprotection and neuronal cell death (Fig 9). The precise events that would tilt this balance toward the latter are currently being explored. It is interesting to note that PAF, being short-lived and rapidly degraded by PAF acetylhydrolase (16), is a long-term signal with consequences to neurons though COX-2 sustained expression. COX-2 is localized in the nuclear envelope and perinuclear endoplasmic reticulum. The overexpression of hippocampal COX-2 during cerebral ischemia and seizures may in turn lead to the formation of neurotoxic metabolites (e.g. superoxide). Current investigations aim to determine whether or not other messengers cooperate to enhance neuronal damage (e.g. nitric oxide) and the possible involvement of astrocytes and microglial cells. Further understanding of these

potentially neurotoxic events involving lipid messengers and COX-2 will permit the identification of new strategies and define therapeutic windows for the management of epilepsy.

8. *Acknowledgments*

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Figure Legends

Figure 1.

Sites of phospholipid cleavage by the major classes of phospholipases, showing some of the important reaction products. The top two reactions show the phospholipase A_2-catalyzed degradation of choline phospholipids. In the first reaction, the C2 arachidonoyl chain of PC is cleaved to liberate free arachidonic acid (AA). This reaction is probably the quantitatively most important source of free AA for the synthesis of eicosanoids. The second reaction involves the quantitatively much less significant ether lipid analogue of PC, alkyl arachidonoyl GPC. Although this reaction probably does not contribute significantly to overall AA release, the lysophospholipid formed is the direct precursor of the highly potent platelet-activating factor. The third reaction shows the action of inositol phospholipid (PI)-specific phospholipase C on PIP_2. This classical bifurcating signaling reaction yields DAG, an activator of protein kinase C, and IP_3, a mobilizer of intracellular calcium stores. The bottom reaction shows the action of phospholipase D on PC. The PA released can provide a source of DAG quantitatively much more significant than that derived from the smaller PI pool, and is also a precursor of the novel bioactive lipid lyso-PA. The free choline released is thought to be an imporatnt source of precursor for the neurotransmitter acetylcholine.

Figure 2.

Summary of the pathways of PAF synthesis and degradation. The central portion of this

figure represents the "PAF cycle" between the membrane phospholipid PAF precursor alkylacyl GPC and biologically active PAF. The remodeling synthesis involves the production of lyso-PAF, generated from the PAF precursor alkylacyl GPC either by the direct action of phospholipase A_2 or by the transfer of the *sn*-2 acyl chain to a "donor" lysoplasmalogen, which is itself mobilized from membrane plasmalogen by phospholipase A_2 action. The de novo pathway involves the direct transfer of a choline moiety to alkyl-acetylglycerol. Note that PAF acetylhydrolase inactivates all PAF molecules regardless of their biosynthetic route, and additionally inactivates oxidatively damaged phospholipids that exhibit biologic activity at the PAF receptor.

Figure 3.

Schematic representation of the proposed role of PAF in the regulation of excitatory neurotransmitter release and how, during cerebral trauma, it may play a role in the excessive release of neurotransmitter. Under normal physiological conditions, glutamate is released from a presynaptic terminal and stimulates NMDA receptors at the postsynaptic terminal. The resultant elevation of intracellular calcium activates cytoplasmic PLA_2 and initiates accumulation of PAF and 20:4. Upon sufficient stimulation there is enough PAF to diffuse back across the synapse and provide a sufficient concentration at the presynaptic terminal to stimulate the BN 52021-sensitive, presynaptic PAF receptor and, via a mechanism as yet undefined, stimulate glutamate release. At the same time, 20:4 accumulates in the postsynaptic terminal and have an

positive effect on glutamate neurotransmission involving the NMDA receptor. Under pathophysiological conditions such as during cerebral ischemia, glutamate neurotransmission could be elevated by a superinduction of these pathways where an initially elevated signal at the presynaptic terminal could initiate a positive feedback loop.

Figure 4.

Time course of changes in relative mRNA abundance and transcriptional activity in hippocampus after KA treatment or a single electroconvulsive shock (ECS).

(A) Relative abundance of COX-2 and *zif*-268 mRNAs compared to GAPDH mRNA, as assessed by northern analysis. (n=9-12 for each time point from 3 separate experiments, error bars ± 1 s.d.)

(B) Transcriptional activity of COX-2, COX-1 and *zif*-268 genes assessed by nuclear run-on transcription. (n=3-4 from 3 separate experiments). Data are normalized to transcriptional activity of GAPDH.

Figure 5.

Prostaglandin endoperoxide synthase-2 and *zif*-268 in hippocampus: Time course of changes in relative mRNA abundance and transcriptional activity in hippocampus after KA treatment.

(A) Relative abundance of COX-2 and *zif*-268 mRNAs compared to GAPDH mRNA, as assessed by northern analysis. (n=9-12 for each time point from 3 separate experiments,

error bars ± 1 s.d.). (B) Transcriptional activity of COX-2, COX-1 and *zif*-268 genes assessed by nuclear run-on transcription. (n=3-4 from 3 separate experiments). Data are normalized to transcriptional activity of GAPDH.

Figure 6.

Time course of COX-2 and COX-1 protein accumulation in rat hippocampus after kainic acid injection. (A) Representative Western blots of hippocampal protein extracts using COX-2 (black circles) and COX-1 (open circles) antibodies. The positive control for COX-2 (ϕ) is from lipopolysaccharide-induced macrophages. (B) Quantification of Western blots to assess relative induction of COX-2 and COX-2 protein in experimental (kainic acid treated) vs. control (saline-treated). (n=8-12, from 3 separate experiments, error bars ± 1 s.d.)

Figure 7.

Inhibition by BN 50730 pretreatment of KA-induced COX-2 protein accumulation (open bars), but not of endogenous COX-1 (closed bars) in rat hippocampus.

(A) Representative Western blots. BN 50730 or vehicle treatments were as for Fig. 4. Samples were collected 6 hours after KA treatment.

(B) Quantification of Western blot data expressed as a percent increase over control (vehicle-pretreated) values. (n=10-12 from 3 separate experiments, error bars ± 1 s.d.)

Figure 8.

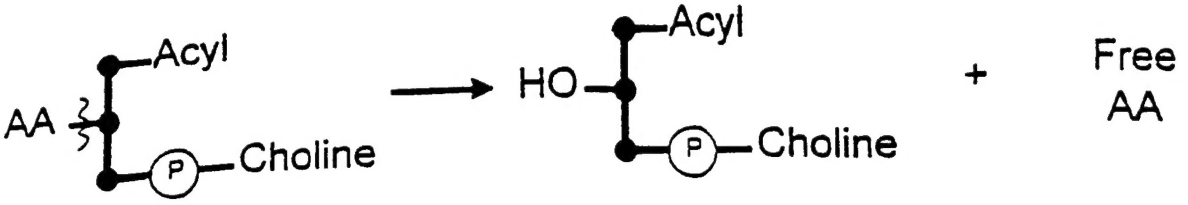
Seizure-triggered signaling events linking synapse activation and COX-2 gene expression in neurons. NMDA-receptor activation by glutamate leads to phospholipase A_2 activation and the generation of PAF and of arachidonic acid. PAF is synthesized through other metabolic routes as well. PAF activates COX-2 gene expression through a BN-50730-sensitive intracellular site, protein kinase cascades and transcription factors. The COX-2 promoter is also a target for cytokines (activation) and glucocorticoids (inhibition). COX-2 protein then catalyzes the conversion of arachidonic acid into PGH_2, the precursor of eicosanoids. Constitutive COX-1 also catalyzes this metabolic step (modified from N. Bazan, *Primer Cerebrovascular Diseases*. M Welsh, L Chaplan, D Reis, B Siesjö, B Weir (eds), Academic Press, 1997, with permission).

Figure 9.

$cPLA_2$ and the generation of bioactive lipids during ischemia and reperfusion. During the ischemic phase, phospholipase overactivation and the down-regulation of oxidative and energy metabolism, and hence reincorporation of $cPLA_2$ metabolites, promote the accumulation of AA and lysophospholipids such as lyso PAF. The reperfusion state permits the completion of PAF and eicosanoid synthesis, but at the expense of the accumulation of pathophysiologically high levels of these mediators. Reactive oxygen radicals are generated at rates that can overload the antioxidant and free radical scavenger systems of the brain, thus allowing free radical damge to a range of molecules,

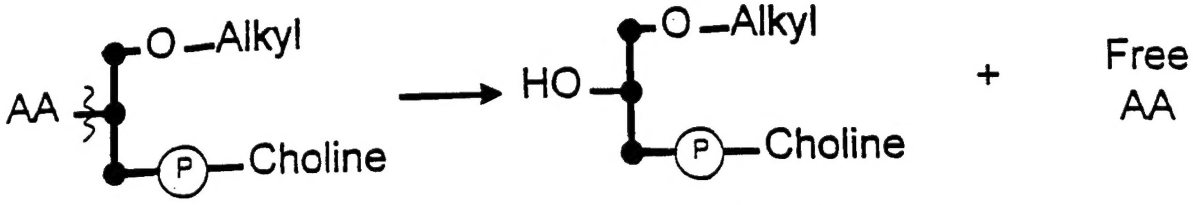
including peroxidation of polyunsaturated fatty acids.

Phospholipase A_2



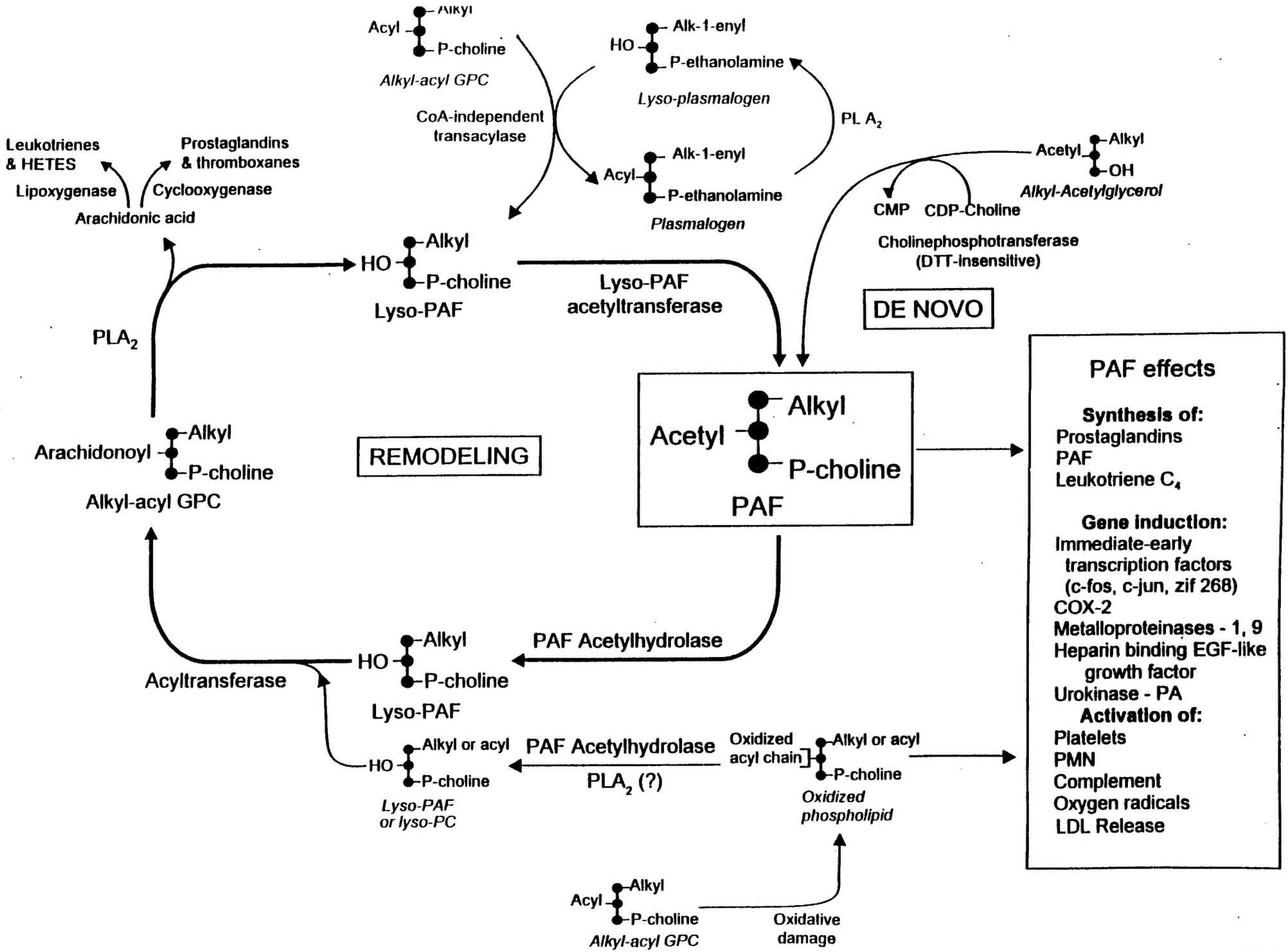
1-acyl-2-arachidonoyl
-glycero-3-phosphocholine
(PC)

lyso-PC



1-O-alkyl-2-arachidonoyl
-glycero-3-phosphocholine
(Platelet-activating factor precursor)

lyso-Platelet
-activating
factor


Alkyl
Acyl
P-choline
Alkyl-acyl GPC
CoA-independent transacylase
Alk-1-enyl
HO
P-ethanolamine
Lyso-plasmalogen
PL A2
Alk-1-enyl
Acyl
P-ethanolamine
Plasmalogen
Leukotrienes & HETES
Prostaglandins & thromboxanes
Lipoxygenase
Cyclooxygenase
Arachidonic acid
Acetyl
Alkyl
OH
Alkyl-Acetylglycerol
CMP
CDP-Choline
Cholinephosphotransferase (DTT-insensitive)
HO
Alkyl
P-choline
Lyso-PAF
Lyso-PAF acetyltransferase
DE NOVO
PLA2
Arachidonoyl
Alkyl
P-choline
Alkyl-acyl GPC
REMODELING
Acetyl
Alkyl
P-choline
PAF
PAF effects
Synthesis of:
Prostaglandins
PAF
Leukotriene C4
Gene induction:
Immediate-early transcription factors (c-fos, c-jun, zif 268)
COX-2
Metalloproteinases - 1, 9
Heparin binding EGF-like growth factor
Urokinase - PA
Activation of:
Platelets
PMN
Complement
Oxygen radicals
LDL Release
PAF Acetylhydrolase
Acyltransferase
HO
Alkyl
P-choline
Lyso-PAF
Alkyl or acyl
HO
P-choline
Lyso-PAF or lyso-PC
PAF Acetylhydrolase
PLA2 (?)
Oxidized acyl chain
Alkyl or acyl
P-choline
Oxidized phospholipid
Acyl
Alkyl
P-choline
Alkyl-acyl GPC
Oxidative damage